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C.-E. A. WINSLOW

It is characteristic of Science and Progress that they continually open new fields to our visions.—PASTEUR.

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CERTAIN GENERA OF THE CLOSTRIDIACEAE

STUDIES IN PATHOGENIC ANAEROBES. V

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In a previous paper a classification was suggested for the group of anaerobic rods which include, roughly, the anaerobic members of the genus *Bacillus* of former workers. A family was proposed for these organisms, with the name *Clostridiaceae*,[1] and it was divided into two subfamilies, the *Putrificoideae*[2] or proteolytic anaerobes, and the *Clostridioideae*[2] or non-proteolytic anaerobes. Certain genera for these groups are proposed in the present communication. These in some cases unite various described species, while in other cases the genera themselves correspond to the former idea of species.

A key to the genera is also given, which is based on the action of the anaerobes on meat medium and on their general cultural behavior and morphology. Possibly the main lines for tribal organization will to some extent follow this key, but it pretends to be nó more than an artificial arrangement. It is in no way complete and cannot be implicitly relied upon for purposes of classification. It is meant more as an index to the forms whose descriptions are sufficiently clear to warrant assigning them definite positions. The key will serve, for a time, as a nest of pigeonholes in which to place new species until material is sufficient for a complete reorganization, but workers should not try to place all newly discovered organisms in these genera.

The conservative worker, familiar with aerobic pathogens, who enters the anaerobic field, is all too prone to wish to "identify"

[1] For definition see Jour. Bact., **6**, 536.

[2] For definition see Jour. Bact., **6**, 550.

his anaerobic strains. A natural feeling exists among systematists, that the description of a new-found type as a new species is to be avoided unless the describer is very sure that his type differs from all others. There is a fear that a description carefully made will some day be discarded by a future taxonomist who finds it identical with a former description. This logical attitude has bred and fostered a wholly unscientific mania for identification of all new strains with organisms already described by others. In the case of parasites or in well-known groups this procedure will only occasionally lead the worker astray. In the anaerobic field it will do so frequently. The scientific attitude relative to the taxonomic affinities of anaerobic bacilli is to state the group or generic relationships of the organisms and then to describe them minutely. The routine bacteriologist should be content to assign an organism to its proper genus.

Colony formation in deep agar is included in the descriptions of genera, but a restriction of the genera to such types of colony form as are mentioned would be unwise. The definition of the Gram staining reactions should not be regarded as of great value in these descriptions, as the staining reactions of the organisms and the technique of workers are too variable. Sugar fermentations may be relied upon in the case of actively growing species only, and are valid only for media on which the behavior of a strain is constant. The constancy of the fermentations here quoted has not been verified by myself. Pathogenicity is not to be taken as a criterion for admission to a genus.

It is to be feared that bacteriological systematists who are unacquainted with the anaerobic group will object to the creation of so many genera as are here proposed. The Committee on Characterization and Classification of Bacterial Types (1920) propose but 38 genera for the orders *Actinomycetales* and *Eubacteriales*. The influence which the medical history of the science of bacteriology has had on this classification is marked. Two-thirds of the 38 genera recognized contain parasites. If we regard the anaerobic bacteria as wild plants growing in soil, as we have every reason for doing because of their many species, we

must classify them as do the botanists and not as do the bacteriologists. The family of *Orchideae* has 334 genera, the *Gramineae* 298, the *Rubiaceae* 377, the *Leguminoseae* 399, and the *Compositae* 766 (Bentham and Hooker). The following classification imitates the taxonomic arrangement of botanical classifications in preference to bacteriological. It must be borne in mind that the date 1920 in bacteriologic systematics corresponds roughly to the date 1760 in botanical systematics.

Relative to the evolutionary history of the anaerobes we know nothing, but conjectures are not entirely out of place. Proteins and carbohydrates have existed together and have been destroyed by bacterial action since a remote geologic epoch. We cannot tell whether the organic catalyzers of one type of substance antedated those of the other or whether they are closely related. Botanists are not agreed as to what form of life first tenanted the globe. When looking for ancestral forms it is natural to seek in a group of organisms of great catalytic activity like the *Clostridiaceae*, for types which may synthesize simple substances, and if such are discovered it is not out of place to regard them as more closely related to the hypothetical ancestors of the group than those that do not synthesize inorganic substances. Such reasoning is of course entirely dependent on the homogeneity of the group under discussion. We have no means of proving the homogeneity of the *Clostridiaceae*. But when classified according to their chemical activities these organisms form a remarkable chain whose links we are all but warranted in regarding as the varied end points of an evolutionary process. As our most primitive type we may well choose the nitrogen-fixing anaerobes of the soil. It is to Winogradsky that we turn for the first demonstration of such organisms. He named his anaerobic nitrogen-fixing organism *Clostridium Pastorianum*. Bredemann declares that nitrogen-fixation is a variable power among all organisms of the amylobacter type, and he includes under the name amylobacter, *Clostridium Pastorianum* of which bacillus he possesses a strain. These organisms are highly saccharolytic, being pectin fermenters, and they do not split gelatin. Farther up the scale we find anaerobic rods which

are actively saccharolytic and do split gelatin, for example the Welch bacillus, vibrion septique, and many other forms; these organisms do not liquefy coagulated serum or produce hydrogen sulfide or other protein split products in any considerable quantity. Most of them possess no diastatic power toward pentosans, and many fail even to split pentoses. The group next in the scale are those anaerobes which, though they fail to disintegrate coagulated serum and muscle particles, are sufficiently proteolytic to free considerable quantities of hydrogen sulfide in media rich in sulfur, such as blood broth. The oedematiens type, and other less well-known organisms may be placed here. Some of these are strongly saccharolytic and others are weakly so. Further advances in attack on the protein molecule are almost invariably accompanied by a decrease in saccharolytic power. *B. aerofoetidus*, and the two strains which I term *Reglillus* are apparently slightly proteolytic, and only moderately saccharolytic. The more highly proteolytic organisms usually split monohexoses, glucose only, or no sugars at all. The most highly adapted catalytic anaerobes are those that produce both acid and alkali from their substratum in sufficient quantities to keep their hydrogen-ion end point within their optimum range and which are so highly proteolytic that they disintegrate a great variety of protein molecules and split-products. Such types are *B. histolyticus* and *B. botulinus*. Another type, known as *Bifermentans*, keeps its end point within its optimum range of growth but is not sufficiently proteolytic to continue multiplication for a long period. Such organisms are not highly but widely specialized, and are adapted to fend for themselves because they are saccharolytic as well as proteolytic.

It is to render possible a future scientific and logical classification of anaerobic organisms that this key and these generic definitions are proposed. They are manifestly incomplete, and it is certain that careful taxonomic investigations will require the emendation of many of the proposed genera. But the present paper aims to blaze a trail where meandering paths have wandered—a trail upon which a highroad may be built in the future.

I wish most heartily to thank Dr. Karl F. Meyer for many helpful suggestions which he has given me during the compilation of this classification.

ARTIFICIAL KEY TO THE GENERA OF THE CLOSTRIDIOIDEAE

A. Do not produce H_2S demonstrable by lead-acetate-paper test when grown in blood-broth.
  B. Do not liquefy gelatin
    C. Very large rods that form oval spores and store up starch in carbohydrate media.
      Genus 1. *Clostridium* Prazmowski emmend. Heller. Type species *butyricum* as described by Winogradsky (1895).
    CC. Rods with spherical spores.
      D. Do not grow on media containing much protein.
        Genus 2. *Omelianskillus* nov. gen. Type species *hydrogenicus* as described by Omeliansky (1895 and 1904b).
      DD. Grow on ordinary anaerobe media.
        E. Sides of the bacilli parallel, spores strictly terminal.
          Genus 3. *Macintoshillus* nov. gen. Type species *tetanomorphus* (pseudotetanus bacillus, McIntosh (p. 32), *Bacillus tetanomorphus* Committee (p. 41)), as described by the Committee.
        EE. Spores not always strictly terminal, sides of bacilli may not be parallel.
          Genus 4. *Douglasillus* nov. gen. Type species *sphenoides* (*Bacillus sphenoides* Douglas Fleming and Colebrook), as described by the Committee (p. 43).
    CCC. Slender rods with oval endspores, usually Gram-negative.
      D. Clot milk and attack various sugars, produce much acid.
        Genus 5. *Henrillus* nov. gen. Type species *tertius* (*Bacillus tertius* Henry) as described by Henry.
      DD. Do not clot milk, attack few or no sugars, produce little acid.
        Genus 6. *Flemingillus* nov. gen. Type species *cochlearius* (*Bacillus cochlearius* Douglas Fleming and Colebrook) as described by the Committee (p. 40).
    CCCC. Gram-positive rods which are not markedly slender and which produce oval spores.
      D. Clot milk, saccharolytic.
        E. Sporulate meagerly, attack a few sugars; occasionally moderately pathogenic tissue invaders.

Genus 7. *Vallorillus*. nov. gen. Type species *fallax* (*Bacillus fallax* Weinberg and Séguin) as described by the Committee (p. 27).

EE. Sporulate readily, attack several sugars; not known to be pathogenic.

Genus 8. *Multifermentans* nov. gen. Type species *tenalbus* (*Bacillus multifermentans tenalbus* Stoddard) as described by Stoddard (1919 b).

DD. Do not clot milk. Large Gram-positive rods with long elipsoid spores.

Genus 9. *Hiblerillus* nov. gen. Type species *sextus* (bacillus VI of von Hibler) as described by von Hibler (1908). (Résumé by Weinberg and Séguin (p. 202).)

BB. Liquefy gelatin.

C. Produce stormy fermentation of milk and sporulate on alkaline media only.

Genus 10. *Welchillus* nov. gen. Type species *aerogenes* (*Bacillus aerogenes capsulatus* Welch and Nuttall), type 1 as defined by Simonds (1915 a and b).

CC. Do not produce stormy fermentation of milk.

D. Do not sporulate.

Genus 11. *Stoddardillus* nov. gen. Type species *egens* (*Bacillus egens* Stoddard) as described by Stoddard (1919 a).

DD. Sporulate readily.

E. Gram-positive, form woolly colonies in deep agar. Typically highly pathogenic tissue invaders of many species of animals.

Genus 12. *Rivoltillus* nov. gen. Type species *vibrion* (the vibrion septique of Pasteur) as defined in a future paper.

EE. Gram-negative, may contain Gram-positive granules. Form smooth lenticular or modified lenticular colonies in deep agar. Typically pathogenic for cattle, sheep, and guinea-pigs.

Genus 13. *Arloingillus* nov. gen. Type species *Chauvoei* (*Bacterium Chauvoei* Arloing, Cornevin and Thomas) as described in a future paper.

AA. Produce H_2S demonstrable by a lead-acetate-paper test when grown in blood broth.

B. Produce a large amount of gas from carbohydrates. Heavy Gram-positive rods with little or no tendency to sporulation.

Genus 14. *Meyerillus* nov. gen. Type species *sadowa* nov. sp. to be described in a future paper.

BB. Produce less gas from carbohydrates. Heavy Gram-positive or Gram-negative rods that form oval spores that are usually subterminal.

Genus 15. *Novillus* nov. gen. Type species *maligni* (*Bacillus oedematis maligni II.* Novy) as described by Novy.

ARTIFICIAL KEY TO THE GENERA OF THE PUTRIFICOIDEAE

A. Produce an alkaline reaction in meat medium but do not grossly disintegrate the particles of meat.

B. Minute Gram-positive rods which form few spores. Clot milk. Weak tissue invaders, or non-pathogenic.

Genus 16. *Seguinillus* nov. gen. Type species *aerofoetidus* (*Bacillus aerofoetidus* Weinberg and Séguin) as described by Weinberg and Séguin (p. 161).

BB. Gram-positive rods with oval subterminal or median spores that do not greatly distend the sides of the bacilli. Do not ferment milk. Typically pathogenic tissue-invaders.

Genus 17. *Reglillus* nov. gen. Type species *progrediens* nov. sp. to be described in a future paper.

AA. Attack proteins somewhat more energetically than the above and produce a terra cotta coloration of the meat particles and soften and partially disintegrate them. (The terra-cotta color is not to be confused with the pink color produced in meat medium by the acid from sugar fermentation.) These organisms continue to multiply in meat medium at a moderate rate for months.

B. Show no blackening of the meat-particles on prolonged incubation.* Gram-negative or weakly Gram-positive rods with oval subterminal or median spores that distend the bacilli.

Genus 18. *Robertsonillus* nov. gen. Type species *primus* (Bacillus I. Hempl) as described by Hempl.

BB. Show after approximately two weeks' incubation a blackening of some of the meat-particles. Gram-negative rods with spherical end-spores. Typically produce a neuro-toxin.

Genus 19. *Nicollaierillus* nov. gen. Type species *tetani* (*Bacillus tetani* Nicollaier) to be described in a future paper.

---

* "Incubation" refers to anaerobic incubation at 37°C except in the case of deep agar tubes which are incubated in air at 37°C. Meat medium is pH 7.2 to start with.

AAA. Highly proteolytic on meat medium for a short period. Produce a gray coloration or a slight blackening of the medium. Sporulate at an early stage and after three days' incubation vegetate very slowly indeed without so digesting the meat particles that they greatly decrease in size.

B. Thick rods with oval or oblong spores which are usually central and do not greatly distend the bacillus.

C. Resistant saprophytes. Occasionally invade tissue in company with other organisms or alone in debilitated individuals.

Genus 20. *Martellillus* nov. gen. Type species *bifermentans* (*Bacillus bifermentans sporogenes* Tissier and Martelly) as described by Tissier and Martelly.

CC. Delicate parasites. Gram-negative tendency.

Genus 21. *Recordillus* nov. gen. Type species *fragilis* nov. sp.

AAAA. Organisms highly proteolytic, producing on three days' incubation in meat medium partial destruction of the meat particles which continues on further incubation till the meat particles have greatly diminished in bulk. Coloration of meat usually dark brown or terra-cotta; blackening may or may not take place.

B. Slender rods with terminal oval spores.

C. Split sugars.

Genus 22. *Tissierillus* nov. gen. Type species *paraputrificus* (*Bacillus paraputrificus* defined by Bienstock 1906) as described by McIntosh under the name of *Bacillus putrificus* (p. 39).

CC. Do not split sugars.

Genus 23. *Putrificus* nov. gen. Type species *Bienstocki* (*Bacillus putrificus* Bienstock) as defined by Bienstock (1906)).

BB. Heavy rods with subterminal or median oval spores.

C. Do not produce balls of amino-acid crystals to a striking degree.

D. Not so highly proteolytic as organisms of following groups. Meat particles not much decreased in size, spores often larger, and more nearly spherical than in following genera. Isolated colonies in deep agar usually, but not always, large, smooth lenticular or modified lenticular structures that do not become woolly. Typically produce a powerful neuro-toxin.

Genus 24. *Ermengemillus*, nov. gen. Type species *botulinus* (*Bacillus botulinus* van Ermengem) as described by Meyer and co-workers in a future paper.

DD. Highly proteolytic, blacken meat readily, produce penetrating foul odor. Form in deep agar large woolly or spherical colonies or lenticular colonies that show a tendency to become woolly. Are not pathogenic in pure culture.

Genus 25. *Metchnikovillus* nov. gen. Type species *sporogenes* (*Bacillus sporogenes* Metchnikoff) as described by the Committee as Metchnikoff's race A (p. 36).

CC. Produce balls of amino-acid crystals in meat after a comparatively short period of incubation. Violently proteolytic, may produce an exotoxin, and invade tissue, which is vigorously digested.

Genus 26. *Weinbergillus* nov. gen. Type species *histolyticus* (*Bacillus histolyticus* Weinberg and Séguin) as described by Henry.

DEFINITIONS OF CERTAIN GENERA OF THE CLOSTRIDIOIDEAE[3]

GENUS 1. *Clostridium* Prazmowski 1880, emmend. Heller. *Clostridioideae* that do not liquefy gelatin. Most species cause stormy fermentation of milk. Highly saccharolytic, many even splitting pectins. Produce considerable amounts of butyric acid as a split-product of carbohydrate fermentation. May fix nitrogen. Readily derive their nitrogen from inorganic nitrogen salts. Large rods which are frequently polymorphic and form large orgonts and oval spores. Frequently store up starch. Form in deep agar large lenticular or modified lenticular colonies. Common destroyers of plant tissue (not cellulose) in soil. Used in the retting of flax to split pectins.

Type species, *C. amylobacter* van Tieghem as described by Winogradsky (1896). Probable synonyms: *Amylobacter* Trécul, *Clostridium butyricum* Prazmowski, *Amylobakter* Gruber, *Granulobakter saccharo-butyricum* Beijerinck, *B. saccharobutyricus* von Klecki, *Granulobacillus saccharobutyricus mobilis nonliquefaciens* Grassberger and Schattenfroh, *B. amylobacter* von Hibler, *B. amylobacter* Arthur Meyer and Bredemann. Most of these are today incapable of accurate definition and several of them represent groups and not entities. For this reason the type description chosen is that of Winogradsky instead of that of Prazmowski.

[3] For definition see Jour Bact., **6**, 550.

Trécul (1865) gave the bacteria that contain starch the name of *Amylobacter*. He (1867) declared the *Amylobacter* to be heterogenetic, formed of minute particles that organize themselves into bacilli in decaying plant tissue. Van Tieghem (1877) named *Bacillus amylobacter* bacilli which contained amorphous starch during their growth stage. He believed such organisms to be the agents of cellulose destruction. Prazmowski (1880) described and figured *Clostridium butyricum*, which though it was in impure culture, was evidently of the type described above. Winogradsky (with Fribes) (1896) first defined a type that can be considered a species; he assigned no name to the organism. He declares that it does not split cellulose, but pectin. It ferments glucose, sucrose, lactose and starch in peptone media.

This type of organism has been described by many workers. It has been most thoroughly discussed by Bredemann. *Bacillus amylobacter* A. M. et Bredemann probably includes all the large starch-storing clostridia described above in the generic definition. In his investigation Bredemann used principally cytological criteria, and essential extensive chemical studies were not made. He regards all differences noted between his strains as fluctuating variations. It would seem illogical from the point of view of the general systematist, to assign merely specific rank to a group of organisms so widespread and of such abundant occurrence as are these soil anaerobes. Bredemann's investigation simply indicates that he did not find means of distinguishing his strains, or perhaps that by his technique he isolated only a restricted group of the general type. Bredemann declares that the power of fixing nitrogen varies in these organisms and cannot be used as a specific character. Bredemann's critique of his cultures is apparently very weak. Thus on page 404 he claims to have changed a Welch bacillus into an amylobacter. He quotes seriously the fantastic conceptions of Grassberger and Schattenfroh with regard to the "denaturing" of anaerobic organisms, and his conception of systematic anaerobic work is that of Lehmann and Neumann. He regards as a variant of *B. amylobacter* what appear to be coccus forms contaminating his cultures.

The chemical activities of this type of anaerobe with regard to end-products are described in some detail by Bredemann and by Grassberger and Schattenfroh. The latter find that amylase is usually present, sucrase very rarely so.

Clostridium Pastorianum is the name given to the anaerobic nitrogen-fixing bacillus discovered by Winogradsky (1896 and 1902). This author distinguishes it from other clostridia known to him by the fact that the sporangia only partially disappear from about the spores, forming what he terms a "spore capsule," and by the fact that its fermentative ability is less than is that of most soil clostridia. In peptone media it splits glucose, sucrose, laevulose, inulin, galactose and dextrin, but not lactose, arabinose, starch, rubber, mannitol, dulcitol, glycerol or calcium lactate. Presumably stormy fermentation of milk does not then take place. Obviously the production of stormy fermentation of milk, depending on the splitting of one sugar, is not to be regarded as a generic character. Grassberger and Schattenfroh found certain strains of their organism which did not attack milk with energy. *Clostridium Pastorianum* is sufficiently well differentiated by Winogradsky from the ordinary *amylobacter* or *butyricum* type to warrant its separation from that type as a separate species. Bredemann regards the "spore capsule" formation described by Winogradsky as a frequent anaerobe character. I have never seen an anaerobe strain which produced the remarkable "spore capsules" figured by Winogradsky. This author isolated *C. Pastorianum* only a few times out of many samples of earth, and he was familiar with the type usually termed *C. butyricum* or *B. amylobacter*.

Gruber distinguished two types of sporulating anaerobic granulose-storing butyric acid bacilli, of which the first is the most like the usual conception of *C. butyricum*.

Beijerinck differentiated his granulobacilli into an anaerobic and an aerobic form: Grassberger and Schattenfroh were unable to confirm this work.

Choukevitch (1911) distinguished three types of amylobacter: one fermented glucose, lactose, starch and hemicellulose; one glucose and lactose only; and a third rarely stored up starch

and had little fermentative power. He (1913) identified as *B. amylobacter* strains which ferment cellulose.

Pringsheim described *C. americanum*, which fixes nitrogen less energetically than *C. Pastorianum* and ferments the same sugars as that organism, besides mannitol, glycerol and lactose. It will grow in open flasks; Bredemann terms it an anaerobe.

Douglas, Fleming and Colebrook have described under the name *B. butyricus* a medium sized bacillus that should be assigned to this genus or to *Multifermentans*. McIntosh describes what may be the same strain.

Prazmowski described under the name *Clostridium polymyxa* an aerobic organism. The generic name has since been used occasionally for aerobes. It must in future be restricted to the group of anaerobic organisms which most strongly resemble the first type described by Prazmowski, *C. butyricum*.

There apparently remains abundant critical chemical and systematic work to be done in the study of this important genus, and such work should be performed with improved technique and with cultures whose purity will stand criticism.

GENUS 2. *Omelianskillus* nov. gen.

Clostridioideae that do not grow well in media containing much protein, and may derive all their nitrogen from mineral salts. Split cellulose or hemicellulose. Do not contain starch. Long slender bacilli with spherical spores. Colonies may be produced on potato slants; they are minute, yellowish and transparent. Agents of plant putrefaction, found everywhere.

Type species *O. hydrogenicus* (the ferment of cellulose which produces hydrogen, of Omeliansky) as described by Omeliansky (1895 and 1904 b). Characters of genus. Probably several species were studied by Omeliansky. In his later work he admits that the cultures studied by him were not pure. Another type, *O. methanicus*, similar to the above, is said to be the agent of methane formation in cellulose fermentation. This species may be assigned to the same genus.

These organisms were isolated by growing them in a medium free of organic nitrogen. There may be other genera of anaerobic

cellulose splitters which require some organic nitrogen for their metabolism.

Choukevitch (1911) describes under the name *B. gazogenes* an organism of active growth habit and of very strong fermentative powers, that splits starch and hemicellulose. Its morphology is similar to that of Omeliansky's organisms and it may be included in this genus.

Choukevitch (1913) considers his type 1 of *B. Rodella III* as similar to *O. methanicus* and type II as similar to *O. hydrogenicus*, but his reasons for so doing are not very sound. These two organisms do not split cellulose.

GENUS 3. *Macintoshillus* nov. gen.

Clostridioideae that do not liquefy gelatin. They produce acid and gas and no putrefaction in meat media. They do not readily attack milk and they ferment few or no sugars. Gram-negative rods with parallel sides and with terminal spherical spores. Colonies in deep agar are small and irregular but not woolly. Frequently found in wounds. Apparently incapable of invading tissue.

Type species *tetanomorphus* (pseudotetanus bacillus, McIntosh (p. 32), *Bacillus tetanomorphus* Committee (p. 41)), as described by the Committee. Glucose and maltose are fermented.

Bacillus tetanoides A of Adamson (1919) is to be assigned to this group. Acid and no gas is produced from glucose and maltose by the majority of Adamson's strains, while one strain showed no fermentative ability. The former type is probably identical with McIntosh's organism.

Choukevitch (1913) describes as type 1 of the bacillus known as Rodella III a slender and highly saccharolytic organism with spherical spores, resembling morphologically Omeliansky's cellulose fermenters. It does not ferment cellulose nor liquefy gelatin and may temporarily be placed here in spite of the fact that it is said to produce hydrogen sulfide. If the organism does not liquefy gelatin it is unlikely that it produces any considerable amount of hydrogen sulfide from protein when in pure culture. This organism is a common intestinal sapro-

phyte of cattle and sheep. A heavier bacillus, type II, is equally difficult to place. It also produces hydrogen sulfide and is highly saccharolytic.

It may be that another genus should be created for highly saccharolytic spherical end-sporing organisms that grow on ordinary media.

GENUS 4. *Douglasillus* nov. gen.

Clostridioideae that do not liquefy gelatin. They produce little gas in meat medium. They may clot milk. Gram-negative bacilli which are frequently fusiform and may show peculiar involution forms. Spherical spores are formed in the bacilli; the rods are usually widened by the spores so that their sides are not parallel. Young spores may not be truly spherical. Occasionally found in wounds. Probably incapable of invading tissue.

Type species *sphenoides* (*Bacillus sphenoides* Douglas, Fleming, and Colebrook), as described by the Committee (p. 43): the type which ferments glucose, maltose, galactose, lactose, salicin, mannitol, sucrose, dextrin and starch.

The Committee states that the fermentation reactions of this group are variable. Bacillus E of Adamson (1919) is apparently closely related to *D. sphenoides*. It produces peculiar involution forms and though it ferments several sugars (glucose, maltose, lactose and mannitol) it produces very little gas from them, and has not strong fermentative powers.

The pointed rod named *Coccobacillus proeacutus* by Tissier may be included in this genus. It does not ferment lactose or sucrose but does attack glucose.

These organisms are not to be confused with the nonsporulating anaerobic fusiform bacilli which show a spotted staining reaction and invade tissue, but grow poorly on artificial media. These latter may be trained to an aerobic habit. They have been placed in the genus *Fusiformis* in the family *Mycobacteriaceae*, order *Actinomycetales* by the Committee on Characterization and Classification of Bacterial Types, where they may well be left because of the fact that they are highly adapted parasites.

GENUS 5. *Henrillus* nov. gen.

Clostridioideae that do not liquefy gelatin. They produce acid and gas in meat medium. They clot milk readily and attack many sugars, producing much acid. Gram-negative or gram-positive slender rods with terminal oval spores. Their colonies in deep agar are large, lenticular and opaque. Very common in soil, often found in wounds, do not invade tissue.

Type species *H. tertius* (*Bacillus tertius* Henry) as described by Henry. Henry believes that the conception *B. tertius* applies to a group and not to a species. His nine strains which may be taken as a type split the monoses, bioses, mannose, xylose, starch, dextrin, glycogen, salicin, amygdalin and mannitol.

This type was described by von Hibler with the number IX. Fleming (Bac. Y), Rodella (1902) (Bac. III), Robertson (1916 a), McIntosh, Adamson (1919) and the Committee and Weinberg and Séguin also describe it. Choukevitch (1913) identifies spherical sporulating organisms with Rodella III. The original Rodella III did not clot milk and should perhaps be associated with *Flemingillus*.

GENUS 6. *Flemingillus* nov. gen.

Clostridioideae that do not liquefy gelatin. They produce little gas or acid in meat medium. They do not grow very abundantly in milk or change it. They do not show any marked tendency to split sugars. Slender Gram-negative rods with oval terminal spores. Colonies in deep agar lenticular, may show an areola of fine radiations. Frequently found in wounds, not pathogenic for guinea-pigs.

Type species *F. cochlearius* (*Bacillus cochlearius* Douglas, Fleming and Colebrook) as described by the Committee. This type is highly motile and it split none of the carbohydrates that it was grown in. It was described as bacillus III type C by McIntosh.

Bacillus C of Adamson (1919, p. 380) should be referred to this genus. It is slightly motile. McIntosh's Bacillus III A was also considered by Adamson to belong in such a group as this. It splits glucose and maltose only. We may, until the non-

proteolytic slender end-sporing rods have been carefully investigated, include strains that split a few easily fermentable sugars and do not produce much acid in genus *Flemingillus*, and those that split many sugars and tolerate much acid in the genus *Henrillus*.

Choukevitch describes a number of organisms possessing a similar lack of fermentative ability to *Flemingillus*. Possibly his *B. irregularis* belongs here; *Streptobacillus anaerobicus-magnus* does not resemble this type morphologically, but does chemically.

B. ventriosus of Tissier and *B. gracilis-putidus* of Tissier and Martelly resemble this type. *Coccobacillus oviformis* and *B. capillosus* of Tissier resemble it in fermentative powers but not in morphology.

Genus 7. *Vallorillus* nov. gen.

Clostridioideae that do not liquefy gelatin. They produce gas and acid in meat medium but no digestion. They clot milk slowly and attack various sugars. Rather slender Gram-positive rods with little or no tendency to form spores. Form lenticular colonies, "coeurs jaunes," in deep agar. May invade tissue, producing oedema and gas. Pathogenicity transitory.

Type species *V. fallax* (*Bacillus fallax* Weinberg and Séguin) as described by the Committee (p. 27), the type which ferments glucose, laevulose and maltose.

Henry regards this organism as capable of fermenting many sugars and starch.

Choukevitch (1911) describes under the name *B. bifurcatus gazogenes* a large branching organism which has fermentative reactions similar to *Vallorillus*, but can hardly be included in the genus on account of its unusual morphology.

Genus 8. *Multifermentans* nov. gen.

Clostridioideae that do not liquefy gelatin. Produce gas and acid in meat medium. Clot milk readily, without stormy fermentation. Rather small Gram-positive rods with oval central or subterminal spores. Actively saccharolytic. Found occasionally in wounds, not tissue invaders.

Type species *M. tenalbus* (*Bacillus multifermentans-tenalbus* Stoddard) as described by Stoddard (1915 b). This organism, of which Dr. Stoddard was so kind as to send me a culture, does not fit into any other of the genera here defined. Stoddard's organism ferments glycerol, maltose, lactose, raffinose, glucose, sucrose, inulin, and salicin. Mannitol and dulcitol are not fermented.

Adamson describes under the name *B. butyricus* a "small or medium-sized" bacillus that ferments glucose, lactose, maltose and sucrose but not mannitol and starch. A butyric acid odor is produced. Perhaps it should be assigned to this group.

GENUS 9. *Hiblerillus* nov. gen.

Clostridioideae that do not liquefy gelatin. They do not clot milk. Large gram-positive rods which form more or less reluctantly long ellipsoid spores; they may form orgonts. Colonies in deep agar, small and lenticular or with fine radiations. May be pathogenic for the guinea-pig, producing oedema and gas, or paralysis.

Type species *H. sextus* (*Bacillus VI* of von Hibler), as described by von Hibler (résumé by Weinberg and Séguin, p. 202).

Von Hibler describes another species which he terms VII. These organisms have much in common and we are probably justified in including them in one genus as *Hiblerillus sextus* and *Hiblerillus septimus*. The latter resembles an organism described by Tizzoni and Cattani, according to von Hibler. There are probably many organisms in soil which are pathogenic for rabbits and guinea-pigs when given certain conditions favorable to invasion, which rarely invade under natural conditions or which on account of shy growth habit are missed when they invade in the company of other organisms.

To this genus may be assigned two organisms isolated from the intestine of the horse and described by Choukevitch (1911); they possess similar fermentative ability: the production of acid and no gas in glucose agar. A non-pathogenic one, *Streptobacillus anaerobicus-rectus* may be termed *H. rectus*; another, *H. megalosporus*, produced a fibrino-purulent peritonitis in a guinea-pig.

GENUS 10. *Welchillus* nov. gen.

Clostridioideae that liquefy gelatin but do not produce hydrogen sulfid demonstrable by lead-acetate-paper test in blood-broth. They produce much acid and gas on meat medium, but they do not digest it, nor do they digest casein, coagulated serum, or eggwhite. They produce stormy fermentation of milk and attack many sugars vigorously. Their multiplication is extraordinarily rapid; they are killed by their own growth products in acid media, in which they fail to sporulate. Deeply Gram-positive non-flagellate rods with square ends. They produce oval subterminal or median spores in alkaline media, or in media free of fermentable carbohydrate; these spores do not bulge the sides of the bacilli. When growing rapidly the rods are very short, and resemble closely no other type here listed except *Stoddardillus*. When growing slowly the rods are less abundant and longer, and may be mistaken for *Martellillus* and similar organisms. Typically intestinal saprophytes; ubiquitous. Many strains produce toxin and invade tissue, forming gas, and causing the formation of oedema, and in many cases causing the disintegration of muscle and of connective tissue without the production of a foul odor. This disintegration occurs only *in vivo*, and is probably due to the enzymes of the host tissue. *Welchillus* are the most frequent anaerobic invaders noted on the hospital autopsy table: the organisms are present in human intestines. Causative agents of a probably greatly overestimated percentage of gas gangrene cases following war wounds. Comparatively rare as animal invaders.

Type species *W. aerogenes* (*Bacillus aerogenes-capsulatus* Welch and Nuttall) type I as defined by Simonds (type IV of Esty). Ferments, besides other carbohydrates, inulin and glycerol. Usually pathogenic for guinea-pigs.

Synonyms. B. phlegmones-emphysematoseae Fraenkel, *B. perfringens* Veillon and Zuber, 'Bacille de reumatisme aigue' of Achalme, *Butyribacillus immobilis-liquefaciens* Grassberger and Schattenfroh. Descriptions and discussions, most of them with large bibliographies, are to be found under the names of the following authors: von Hibler, Simonds, Robertson, Wein-

berg and Séguin, Henry, McIntosh, Adamson, The Committee, Jablons, Esty. Simonds divided the group into four sub-groups on the basis of the fermentation of inulin and glycerol. Henry and Esty substantiated this finding. The latter finds his strains divisible into two sub-groups on the basis of the sensitiveness of the spores to heat. The division thus made does not coincide with those secured by means of sugar fermentation. McIntosh admits a species which sporulates on ordinary media. The Committee, on which McIntosh later served, do not mention such a type. The chemical behavior of *Welchillus* has most recently been studied by Wolf (1919) and Wolf and Harris (1917, a, b, and c). Agglutinins are extremely difficult to produce with these organisms, and are found to agglutinate only homologous strains. Werner succeeded in agglutinating one non-homologous strain. Robertson (1916 b) failed to immunize guinea-pigs with bacterial protein. Toxins, according to the work of Bull and Pritchett, are all neutralized by the same antitoxin. Esty immunized guinea-pigs with young whole cultures.

GENUS 11. *Stoddardillus* nov. gen.

Clostridioideae of energetic growth habit that liquefy gelatin, but do not produce H_2S demonstrable by a lead-acetate-paper test in blood broth. Produce abundant gas but little acid in meat medium. Grow very shyly or not at all in milk. Attack a few sugars, but do not produce much acid. Short chunky Gram-positive rods which do not form spores. Colonies in deep agar large, lenticular and opaque. Not easily distinguished from *Welchillus*. May invade tissue, causing considerable destruction of muscle but no foul odor. One strain, reported from a case of human gas gangrene.

Type species *S. egens* (*Bacillus egens* Stoddard) as described by Stoddard (1919 a). Splits glucose, laevulose, mannose, maltose, dextrin, glycogen, inosite and glycerol. Does not sporulate on six days' incubation in inspissated serum.

Such organisms as this are probably not nearly so rare as reports would indicate because they do not sporulate and are

killed in most anaerobic isolation procedures. Because of their close resemblance to *Welchillus* and because of the ubiquity of organisms of that genus their detection is rendered still more difficult.

It may be that later workers will prefer to include this organism in the genus *Welchillus*. The action on milk is dependent on the fermentation of lactose, and sugar fermentations are not to be regarded as of generic significance. I place it in a genus by itself because spore formation has not been demonstrated for this organism.

Genus 12. *Rivoltillus* nov. gen.

Clostridioideae possessing moderately strong saccharolytic powers. Liquefy gelatin but do not produce H_2S demonstrable by a lead-acetate-paper test in blood broth. Produce in meat medium gas and a pink coloration which does not rapidly fade. Clot milk. Do not liquefy serum or egg or disintegrate meat particles. Gram-positive rods, usually short, with median, sub-terminal, or terminal spores, which usually bulge the sides of the bacillus. Sporangia not often much larger than vegetative rods. In tissue the sporangia may be uneven in their staining reactions, "granulose" being present; orgonts are long, frequently with parallel sides. Usually form chains on the liver of animals. Colonies in deep agar, though they may start as lenticular structures, consist later of a dense center and a wide loose woolly periphery. They vary in size, etc., according to species. Typically highly pathogenic tissue invaders that produce haemolysis and gas in the animal body. Pathogenic for a wide range of species.

Type species *R. vibrion* (the vibrion septique of Pasteur), as defined in a future paper. Robertson (1920) has divided the group into four sub-groups on the basis of the agglutination reaction.

Probably the group of anaerobes whose nature is most frequently discussed. The morphological resemblance of individuals and of colonies to organisms of the sporogenes type led to the frequent description of mixed cultures of the two types,

usually under the name *B. oedematis-maligni*. The literature on this group is too extensive to quote. See Ghon and Sachs, Meyer, Weinberg and Séguin, Robertson, the Committee, Wolf (1918). A review of the animal infections has been made by Heller (1920). Under the name *Bacillus tumefaciens* (not to be confused with the plant pathogen *Bacterium tumefaciens*). Wilson describes an organism similar to those of this genus. His description does not convince the reader as to the purity of the culture studied: a mixture of vibrion septique and oedematiens-like organisms would behave as did Wilson's bacillus.

Genus 13. *Arloingillus* nov. gen.

Clostridioideae that attack sugars with considerable energy but have a somewhat restricted action on proteins. Liquefy gelatin but do not produce H_2S demonstrable by a lead-acetate-paper test in blood broth. In meat medium produce gas and a pink coloration that soon fades. Autoagglutinate readily. Clot milk if blood is present. Do not digest serum or egg. Vegetative forms are small gram-negative rods with even staining; forms about to sporulate are uneven in staining reaction, often far larger than vegetative rods, citron or spindle shaped; orgonts (see Heller), show marked tendency to store up granulose. Spores oval, may vary greatly in length. Bacilli do not form long chains on the liver of animals. Colonies in deep agar lenticular, sometimes showing concentric formation, or compound lenticular. Colonies vary considerably according to species. Typically toxic tissue invaders which produce marked haemolysis. Pathogenic for guinea-pigs, cattle and sheep.

Type species *A. Chauvoei* (*Bacterium Chauvoei* Arloing, Cornevin, and Thomas) as described in a future paper.

This genus contains several species which will be discussed. These organisms show some similarity to those of the genus *Clostridium*.

The *B. enteritidis-sporogenes* Klein, as described by von Hibler (1908) should probably be included in the genus. The only character which is markedly different from that of the genus is the energetic fermentation of milk shown by von Hibler's bacillus IV.

GENUS 14. *Meyerillus* nov. gen.

Clostridioideae that produce H_2S on blood broth and liquefy gelatin. In meat medium they produce gas but little or no pink coloration; they show no marked proteolytic action. Do not readily attack milk. Large Gram-positive rods with little or no inclination to form spores. Attack a few sugars. Colonies in deep agar large, opaque and lenticular. Typically tissue invaders of marked power, attacking and digesting *in vivo* the connective tissue more than the muscle.

Type species *M. sadowa* nov. sp. To be described in a future paper. This organism was at first taken for *B. Welchii*. It does not sporulate. It is one of the four guinea-pig invaders isolated from a case of human gas gangrene. I am inclined to place the sporulating bacillus L of Adamson (1919) in this genus but have not handled that organism. Perhaps it is premature to decide whether sporulation may be used as a generic character. Adamson finds bacillus L very slightly proteolytic on milk and not so on other media. *M. sadowa* does not grow on milk.

GENUS 15. *Novillus* nov. gen.

Clostridioideae that in blood broth produce H_2S demonstrable by lead-acetate-paper test; they liquefy gelatin. Produce gas and on continued incubation produce a pink coloration in meat, but this color rapidly fades. Autoagglutinate with extreme readiness. Slowly attack milk and a few carbohydrates, but do not form much acid. Heavy rods of apparently shy growth habit on most media; form a few oval spores that may or may not bulge the sides of the bacilli. Colonies usually large and opaque, may form slender projections or even long fine woolly filaments. Frequently yellowish. Typically toxic tissue invaders which frequently cause the formation of a thick gelatinous oedema that does not lose its gelatinous consistency on section. May also produce gas and a black haemorrhagic condition of the muscle if rapid invasion by large numbers of bacilli takes place. One strain causes considerable tissue destruction. Pathogenic for guinea-pigs, mice, man, hogs, horses, cattle.

Type species *N. maligni* (*Bacillus oedematis-maligni II*, Novy) as described by Novy.

The lesions typically produced by organisms of this group were first described by Koch. Gohn and Mucha give an elaborate description of an organism which belongs to this genus. Von Hibler studied four strains of "Novy's bacillus." Descriptions of organisms of this type have been given by Kerry, by Rivas and by Diedrichs. Weinberg and Séguin described *B. oedematiens* which is different from *N. maligni* but is to be included in this genus. *B. bellonensis* Saquépée also belongs in this genus, as may also the organism called by Adamson bacillus S (1919, p. 373). I have found a type that was rapidly fatal to horses, and another different somewhat from all the rest in a septic wound, and hope to define these species accurately in another paper. Wolf (1920) has studied the biochemistry of an organism of this group.

DEFINITIONS OF CERTAIN GENERA OF THE PUTRIFICOIDEAE[4]

GENUS 16. *Seguinillus* nov. gen.

Putrificoideae that do not exert a marked action on protein. Soften and may blacken meat. Milk clotted, may later be digested. Attack a few sugars. Very reluctant to form spores. Minute Gram-positive or Gram-negative rods, uniform in thickness but not in length. Spores oval, sub-terminal. Deep colonies lenticular or modified lenticular. Occasional tissue invaders which produce a proteolytic gangrene or phlegmon.

Type species *S. aerofoetidus* (*Bacillus aerofoetidus* Weinberg and Séguin) as described by Weinberg and Séguin (p. 161). The organism forms oval subterminal spores.

These may well be called "borderline" organisms. Henry places them in his "saccharolytic group" on account of their sugar-splitting proclivities. A strain given by me by Weinberg does not grossly attack meat particles.

GENUS 17. *Reglillus* nov. gen.

Putrificoideae that show to the eye little proteolytic action on meat. They may or may not blacken it slightly, but the

[4] For definition see Jour. Bact., 6, 550.

meat particles do not diminish in size. Sugars are attacked. Milk is digested. Gram-positive rods which readily form oval subterminal or median spores. Colonies minute, opaque, complex, yellow. Typically toxic tissue invaders which produce in the guinea-pig a clear white oedema that does not rapidly lose its oedematous nature on section.

Type species *R. progrediens* nov. sp. To be described in a future paper. Two species, one found in a case of human gas gangrene (see Barney and Heller), the other in a sheep.

Genus 18. *Robertsonillus* nov. gen.

Putrificoideae that produce on meat medium a little gas and a terra cotta coloration, multiplying slowly for a long period. A black pigment is absent. Sugars not fermented. Weakly Gram-positive or Gram-negative rods with oval spores, usually subterminal, that somewhat distend the bacillus. Two spores often occur in one rod. In old cultures enormous snakey Gram-negative rods are produced. One species, found twice in wounds. Non-pathogenic for guinea-pigs in pure culture.

Type species *R. primus* (Bacillus I, Hempl) as described by Hempl. Descriptions of bacilli similar to this have not been noted. Proteolytic organisms producing a terra cotta coloration in meat medium are not uncommon, however.

Genus 19. *Nicolaierillus* nov. gen.

Putrificoideae that in meat medium produce gas and various color changes: yellowish, pink, grey or mauve, depending on the medium; the particles of meat are gradually suffused with a black pigment, and bleach at the top. The meat is softened but the particles do not greatly diminish in size. Do not attack sugars. Gram-negative (weak methyl violet) rods that form terminal spherical spores. Colonies in deep agar diverse. Common in soil, found in horse feces, may multiply in wounds, but do not normally invade tissue. Produce a characteristic neuro-toxin.

Type species *N. tetani* (*Bacillus tetani* Nicolaier), to be described in a future paper.

Tulloch (1917 and 1919) has divided the group into four types on the basis of the agglutination reaction. Adamson (1920)

describes the cultural behavior of five strains of *B. tetani*. Two papers on the behavior of my cultures are forthcoming.

This is not the genus *Plectridium* of Fischer. *Plectridium* included some butyric acid bacteria, the tetanus bacillus, a putrefactive organism, *Plectridium putrificum*, and other genera not yet known; and it embraces, according to my scheme of classification, elements altogether incoherent. Were the descriptions fuller or were I better acquainted with the proteolytic group, two more genera of proteolytic spherical end-sporers might be suggested: Those that blacken meat readily, e.g., *B. cadaveris-sporogenes*, Klein; and those which do not blacken it at all, e.g., *B. tetanoides B* of Adamson.

GENUS 20. *Martellillus* nov. gen.

Hardy *Putrificoideae* that in meat medium multiply rapidly at an early stage of incubation, producing a greyish coloration and later a blackish deposit on the meat particles, and after three days' incubation cease to multiply actively. Sporulate early in the development of a culture, later cease to do so but vegetate very slowly. Produce very little gas in meat medium. Digest milk. Attack a few sugars. Heavy deeply gram-positive rods, may vary greatly in size. Spores usually cocoon-shaped, usually median or sub-terminal, do not greatly bulge the sides of the bacillus. Colonies in deep agar lenticular, irregular, or stellate. Common putrefactive organisms that readily invade tissue in company with other organisms, producing a greenish proteolytic gangrene. I have found them in the heart's blood and organs of a woman dying of pernicious vomiting and uraemic poisoning and apparently the only invader in a mouse dying from an otherwise unknown cause. The Committee find *B. bifermentans* in acute cases of gas gangrene. Weinberg and Séguin state that such an organism invades guinea-pigs in company with *B. perfringens*. It is possible that *Martellillus* bacilli produce metabolites poisonous to themselves and to animals and that cause early sporulation. They frequently cease active multiplication when the culture has a reaction near pH 7.0.

Type species *M. bifermentans* (*Bacillus bifermentans-sporogenes* Tissier and Martelly) as defined by Tissier and Martelly.

Hempl has described a similar organism not identical with that of Tissier and Martelly (Organism II, which may be renamed *Martellillus proteolyticus*). Bacillus II of Choukevitch probably belongs in this genus, as does von Hibler's bacillus XV. *B. sporogenes B*, as described by Choukevitch also belongs here. A rather shyly growing species sent me by Major Nichols which was named *B. bellonensis* Saquépée, was apparently pure, non-pathogenic, and referable to this genus. It was not the organism described by Saquepee as *B. bellonensis*, nor did it resemble other strains sent me by Saquépée. Adamson describes two species of this type. One under the name of *B. bifermentans-sporogenes*, which is non-motile and does not split sugars, the other under the name of "Central spore bacillus" which he identifies as McIntosh's type XII, which is motile and splits glucose and maltose. The type species is, however, supposed to be saccharolytic. McIntosh's type XII (*parasporogenes*) does not behave in my hands as does this latter type of Adamson's and I should not include them in the same genus. McIntosh's type XIII, a pathogenic proteolytic organism, should probably be placed here. Some of the organisms here listed are probably identical, but one is not warranted in so considering them without a direct comparison of strains. Study of many strains would probably necessitate the division of this genus into two, on the basis of carbohydrate fermentation.

This is one of the important groups that most need systematic investigation.

Genus 21. *Recordillus* nov. gen.

Putrificoideae that, though they sporulate, are exceedingly delicate and soon die in meat medium and other media. Their growth in meat medium resembles that of the organisms of genus *Martellillus;* they do not produce much gas, they color the meat particles a greyish color and form a blackish pigment. Gram-negative rods with central or sub-terminal cocoon-shaped spores. Parasitic forms which infect cattle in California and Nevada. I venture to place such organisms in a separate genus on account of their parasitic habit and on account of their

delicacy. It may be that they are descended from organisms of the genus *Martellillus*.

Type species *R. fragilis* nov. sp. Characters of genus.

I regret that the strain kindly sent me by Dr. Records died soon after its arrival. As it had sporulated in meat medium this was surprising, but was consistent with Dr. Records' description of the behavior of the organism. The strain was isolated from a liver infarct in a cow.

GENUS 22. *Tissierillus* nov. gen.

Putrificoideae that attack sugars and clot milk. Slender Gram-negative or Gram-positive rods with oval terminal spores. Colonies in deep agar have radiate periphery and opaque center. Frequently intestinal saprophytes.

Type species *T. paraputrificus* (*Bacillus paraputrificus* defined by Bienstock) as described by McIntosh under the name *Bacillus putrificus*. Ferments glucose, maltose, lactose, sucrose and starch.

References to organisms of this type are made by Passini, Moro, Metchnikoff and Kligler. Very likely some descriptions of this type were based on the behavior of mixed cultures.

GENUS 23. *Putrificus* nov. gen.

Putrificoideae that do not attack sugars. Slender Gram-negative or Gram-positive rods with oval terminal spores. Colonies in deep agar have radiate periphery and opaque center. Putrefactive organisms found in soil and wounds.

Type species *P. Bienstocki* (*Bacillus putrificus* Bienstock) as defined by Bienstock (1906). References: Bienstock (1884, 1901, 1906), Klein, Rodella (1905). The latter differentiated three types of what he termed *B. putrificus*. Tizzoni, Catani and Baquis described two organisms of this general type. *B. postumus* of Würcker is of this group. His *B. putrificus* is of sporogenes affinities. I possess a strain (IOR) isolated by Miss Robertson or myself from a wound, which is mildly proteolytic, does not split sugars, is Gram-negative, and on serum produces oval terminal spores. Its deep colonies are transparent, len-

ticular, with a protruding fluff. Dr. Meyer tells me that he occasionally encounters such organisms, very slow in initial activity in pure culture, but more active in mixed culture, mildly proteolytic in their action on meat. The colony is not unlike that of *B. botulinus*.

The Committee find that many so-called "*Putrificus*" strains consist of a non-proteolytic oval end-sporer contaminated with a sporogenes-like organism. In the recent German literature "*Putrificus*" refers usually to the sporogenes type. Metchnikoff's strains termed *Putrificus* do not resemble closely those described by Bienstock.

GENUS 24. *Ermengemillus* nov. gen.

Putrificoideae that produce a yellowish coloration in meat medium, and later blacken and digest it. They are more highly proteolytic than the organisms of the foregoing genera. They ferment various sugars. Gram-positive rods which form sub-terminal oval spores. Their colonies in deep agar are, when discrete, lenticular or kidney shaped, and may show tufted smooth or woolly polar projections and infrequently fine loose woolly radiations. Fairly common in soil, grow readily in vegetable material and on meat. Form a characteristic and powerful neuro-toxin.

Type species *E. botulinus* (*Bacillus botulinus* van Ermengem) as described by K. F. Meyer and co-workers in a future paper.

Leuchs discovered that there were two types of toxin produced by different European strains. Burke found two types of toxin produced by different American strains. Probably a number of species may be distinguished by careful methods.

McIntosh and the Committee find that *B. botulinus* shows slight proteolytic power, and meat is stated to be a poor medium for the growth of the organism. My experience has been very different from theirs. Indubitably pure cultures of *Ermengemillus* grow readily on meat medium (pH 7.2) and may show obvious putrefaction in a few days when incubated anaerobically at 37°. But the meat particles are not rapidly diminished in size by the proteolytic action. The observers who did not note the

proteolytic action of these organisms on meat must have used an acid meat medium, or a substratum poor in peptones and peptids, or must have incubated their cultures at room temperature. Von Hibler found that the organism blackened brain medium.

GENUS 25. *Metchnikovillus* nov. gen.

Highly proteolytic *Putrificoideae* that readily blacken meat. They do not produce in it abundant amino-acid crystals, but digest meat, serum, egg and casein rapidly, forming more alkali or less fatty acid than do the organisms of the succeeding group. They split few sugars. Gram-positive or weakly Gram-positive rods, vegetative forms uniform and considerably smaller than sporangia. Sporulate readily in ordinary media, forming oval spores which are usually sub-terminal, though in some strains median spores predominate. Multiplication is exceedingly active, forty-eight hour colonies in deep agar are large and woolly. Frequently a few colonies are larger than the others but they do not give rise in a following generation to a preponderating number of large colonies. Ubiquitous. Common intestinal organisms, abundant in soil; very common in infected wounds. Not capable of invading in pure culture in moderate doses, may invade in company with other organisms or alone when given in large doses.

Type species *M. sporogenes* (*Bacillus sporogenes type A* of Metchnikoff) as described by the Committee (p. 36). Klein described as *B. enteritidis-sporogenes* a mixed culture which contained a non-proteolytic organism, a pure culture of which was described by von Hibler as *B. enteritidis-sporogenes* Klein (von Hibler IV). This tissue invading pathogen, thought by some to have been *B. Welchii*, is most nearly referable to the genus *Arloingillus*. The strain was derived from patients with enteritis and it was apparently contaminated with a proteolytic organism of the genus *Metchnikovillus*. Metchnikoff described two types, A and B, of intestinal anaerobes which he thought were similar to the organism of Klein, and which he termed *B. sporogenes*. His descriptions permit of no identifications. Choukevitch, working in Metchnikoff's laboratory described *B. sporogenes* A and B more carefully. Type B should be referred to genus

Martellillus. Weinberg made the identification of type A more exact, and the Committee discuss it at some length. They allow two species, *B. sporogenes* type A of Metchnikoff, and *B. parasporogenes* McIntosh, which are different in colony formation and serologically. Donaldson describes the "Reading" bacillus, to be assigned to this genus. Superficial acquaintance with many strains of proteolytic anaerobes leads me to suggest that *Metchnikovillus* may be defined as a genus of many species; Dr. K. F. Meyer is also of this opinion. Henry (p. 361) believes it likely that the conception "sporogenes" refers to a group of organisms.

Barger and Dale, Weinberg and Séguin, M. E. Bullock and the Committee discuss the poisonous growth product of this type of organism: it is apparently a lower split-product than are true toxins. Some of these authors find that the culture filtrate of *B. sporogenes* increases the toxicity and invasive power of *B. Welchii*. Donaldson and Joyce placed the Reading bacillus in wounds to digest necrosed muscle and report no accidents due to its presence. Wolf (1919), Wolf and Telfer, and Wolf and Harris (1917, a and b) describe the chemical activities of these and related organisms.

Because of their universal occurrence and active growth habits, organisms of this type frequently contaminate anaerobic cultures. Their chemical activities are thus described in conjunction with the pathogenic properties of the cultures in which they are active. The descriptions which tally more or less accurately with the sporogenes type are legion. Thus *B. oedematis maligni* Koch of von Hibler was probably a mixture of an organism of this genus with a chain-forming vibrion septique; while bacillus XI of von Hibler (1908) was probably such a mixture with a similar pathogen which formed chains somewhat reluctantly (Pl. II, fig. 3). I am today able to discover only the sporogenes type in von Hibler's strain of bacillus XI. Whether bacillus XIX of McIntosh should be included in the genus *Metchnikovillus* is difficult to state. It is apparently an active tissue invader and forms smooth lenticular colonies.

When surface methods of isolation are followed this type of organism is very difficult to remove from cultures of other anaerobes. The Committee emphasize this point. I have had no more contaminations by sporogenes than by aerobes and by various other anaerobes. The organism is common in nature and gains entrance to pure cultures occasionally.

GENUS 26. *Weinbergillus* nov. gen.

Highly proteolytic *Putrificoideae* that in meat medium cause the formation of balls of amino-acid crystals. They digest the meat particles till their bulk is greatly reduced and form large amounts of ammonia, amino-nitrogen and fatty acids. They digest the casein in milk rapidly. Attack few or no sugars. Produce little or no gas in agar media. Gram-positive or Gram-negative rods with sub-terminal oval spores. Colonies in deep agar small, delicate woolly structures. May invade living tissue in company with other organisms, or at times alone, producing a rapid and complete digestion of muscular and connective tissue structures.

Type species *W. histolyticus* (*Bacillus histolyticus* Weinberg and Séguin) as described by Henry (p. 370). Weinberg and Séguin and the Committee allow considerable variation in characters for the strains termed by them *Bacillus histolyticus*.

It is possible that *B. sporogenes-parvus* of Choukevitch may belong in this genus. Wolf and Harris (1918) have made a chemical study of what is probably the type strain—they received it from Henry who received it from Weinberg.

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STUDIES UPON AGGLUTINATION IN THE COLON-TYPHOID GROUP OF BACILLI

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THE PREVENTIVE ACTION OF FORMALIN IN THE AGGLUTINATION TEST

Malvoz in 1897 claimed that formalin produced chemical agglutination of *Bact. typhosum*, but not of *Bact. paratyphosum* and *Bact. coli*, while, on the other hand Beco, Remy, Widal and Nobecourt were unable to confirm Malvoz's observations.

By adding amounts of formalin from 0.05 to 5 per cent to broth or agar cultures, I have obtained no evidences of chemical agglutination with *Bact. paratyphosum* A and B, *Bact. dysenteriae* and *Bact. coli;* with concentrations of 0.05 to 0.3 per cent formalin spontaneous agglutination in these cultures was almost or entirely prevented while *Bact. typhosum* and *Bact. paratyphosum* A showed spontaneous agglutination without formalin but none with it, when the above dilutions were used. When concentrations of 1 to 5 per cent were used slight spontaneous agglutination sometimes occurred. Spontaneous agglutination was more or less inhibited in all dilutions with *Bact. paratyphosum* B. With *Bact. coli* there was absolutely no change in spontaneous agglutination when formalin was added.

From the above facts it is evident that a concentration of 0.05 to 0.2 per cent formalin gives the most satisfactory results in the prevention of spontaneous agglutination, correct reading being possible at the end of two hours. However, it is a better rule to let the tubes stand over night.

Formalin prevents spontaneous agglutination, but does not interfere with agglutination by specific immune sera; it does, in fact, increase the agglutinating reaction, when used with specific serum.

It has been customary to add formalin as an antiseptic, or to prohibit the growth at certain times of the cultures of the colon-typhoid group, which were to be used in conducting the agglutination test. This method was adapted by Loele, who used 2 per cent formalin, while Porges, Coles, Bass and Watkins,

TABLE 1

Standardization of formalin concentration in plain broth culture media to prevent spontaneous agglutination. (Cultures twenty-four hours at 37°C.)

Organism	Bact. typhosum				Bact. paratyphosum A				Bact. paratyphosum B				Bact. coli			
Number of strain	No. 1	No. 2	No. 3	No. 4	No. 1	No. 2	No. 4	No. 6	No. 1	No. 2	No. 3	No. 5	No. 1	No. 3	No. 5	No. 8
Spontaneous agglutination on cultures	4	3	3	3	2	2	3	2	3	3	3	3	3	3	4	4
Dilutions of formalin:																
0.05 per cent	0	0	0	0	0	0	0	0	1	1	1	2	3	4	3	3
0.1 per cent	0	0	0	0	0	0	0	0	1	1	1	2	3	4	3	3
0.3 per cent	0	0	1	0	0	0	1	0	1	1	1	2	3	4	3	3
1.0 per cent	0	0	1	0	1	0	1	0	2	1	1	2	3	4	3	3
5.0 per cent	1	0	1	0	2	1	1	1	2	1	2	2	3	4	3	3
Control no formalin	4	2	3	3	2	2	2	2	3	2	2	3	3	4	3	3

Key: 5, complete; 4, almost complete; 3, weak; 2, very weak; 1, trace; 0, negative agglutination.

Buxton and Vaugham, Rolly, and Asser used 1 per cent, Widal 0.67 per cent, Garrow, Chick 0.1 per cent; Neisser, Proscher, Lion, Martineck, Ehrsam, Flatau and Wilke, and Selter also used formalin and showed, that it had a tendency to slow down or diminish the reaction.

Dreyer has made an especially careful study of the use of formalin. He found that 0.1 per cent formalinized broth cultures agglutinate more actively than those without formalin, and that 0.5 to 1 per cent formalin cultures agglutinate less actively than those without formalin.

In my experiments (table 2) using *Bact. typhosum* and *Bact. paratyphosum* A and specific immune serum, agglutination was strongest with tests containing 0.05 to 0.1 per cent formalin; 0.3 per cent showed slightly less agglutination and 1 per cent

TABLE 2

Standardization of formalin concentration in plain broth culture media to promote specific serum agglutination. (Cultures of twenty-four hours at 37°C.)

Organism	Bact. typhosum						Bact. paratyphosum A						Bact. paratyphosum B					
Number of strains	No. 1	No. 2	No. 3	No. 1, 2	No. 29	Total 5	No. 2	No. 3	No. 6	No. 1, 2	No. 4	Total 5	No. 1	No. 3	No. 5	No. 1, 2	No. 5, 2	Total 5
Spontaneous agglutination on cultures	0	0	0	4	3		0	0	0	2	2		0	0	0	3	3	
Dilutions of formalin and specific serum:																		
0.05 per cent	4	5	5	5	5	24	5	5	5	5	5	25	5	5	5	5	4	24
0.1 per cent	4	5	5	5	5	24	5	5	5	5	5	25	5	5	5	5	4	24
0.3 per cent	4	4	5	4	5	22	5	4	4	5	5	23	5	4	4	5	4	22
1.0 per cent	3	4	4	4	4	19	4	4	3	4	4	19	4	4	4	4	4	20
5.0 per cent	2	3	3	4	3	15	3	3	3	3	4	16	3	3	3	4	4	17
Control, 0	3	4	4	4	4	19	4	4	4	4	4	20	5	4	4	4	4	21
Control without serum:																		
0.05 per cent	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	3	4
0.1 per cent	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	3	4
0.3 per cent	0	0	0	1	0	1	0	0	0	1	0	1	0	0	0	1	3	4
1.0 per cent	0	0	0	1	0	1	0	0	0	1	1	2	0	0	0	1	3	4
5.0 per cent	0	0	0	1	1	2	0	0	0	2	1	3	0	0	0	2	3	5
Control, 0	0	0	0	4	3	7	0	0	0	2	1	3	0	0	0	3	3	6

Keys: 5, complete; 4, almost complete; 3, weak; 2, very weak; 1, trace; 0, negative agglutination.

produced about the same results as those obtained without formalin. With *Bact. paratyphosum* B, agglutination by specific serum in the presence of 0.05 to 0.3 per cent formalin was about the same as, or slightly stronger than, without formalin; with

1 to 5 per cent formalin the agglutination reaction was less than without formalin. However, it has been difficult to standardize this test with formalin, because the reaction depends on the strain of microorganism employed. Many bacilli showed agglutination in all dilutions used and only a few showed different degrees of agglutination; I found that 0.05 to 0.2 per cent formalinized serum gave the best results for most of the strains employed.

TECHNIQUE

Since in plain broth or agar cultures spontaneous agglutination interferes with specific agglutination, in such cultures accurate tests are impossible. When spontaneous and non-spontaneous agglutination types are mixed together, I have observed two types of colonies, (by streak on agar plate) occurring regularly throughout the cultures. One type of colony has a smooth surface and regular outline and shows non-spontaneous agglutination, while the other type of colony has a rough surface and irregular outline and is spontaneously agglutinating.

Either type of organism would, however, produce both types of colonies after growing for some time. Some strains changed in a few days, while others remained true to type, producing characteristic colonies even after continual transplanting for over a year. Cultures used in the experimental work were obtained before each set of experiments by plating and fishing a characteristic colony into experimental media.

Only neutral broth cultures gave uniform results. Therefore 18 to 24 hour cultures in this medium at 37°C., could be used as an indicator of spontaneous and non-spontaneous agglutinations.

The method of my investigations has been to mix 0.3 of each suspension of bacterial cultures in each medium, (such as broth, pepton water and so forth) with 0.3 cc. of the serum dilution, with formalin or without, or with other experimental fluids in small test tubes, of the usual Wassermann type. The tubes, were left at room temperature (excepting in the experiments on temperature), and the preparations were examined, both

macroscopically (by means of a hand lens) and microscopically. In the latter case, as many as 15 or more mixtures were examined at the same time, by transferring a small loopful of each to a square on a large thin glass slide, which had been marked off in squares by means of a grease pencil.

USE OF FORMALINIZED SPECIFIC SERUM IN AGGLUTINATION TEST WITH VARIOUS BACILLI (TABLE 3)

I have observed that specific serum to which has been added 0.2 per cent formalin (final 0.1 per cent) in 0.85 per cent salt solution shows stronger agglutination than occurs without formalin. The formalinized serum mixed with an equal amount of broth culture after three hours at room temperature, shows strong agglutination with *Bact. typhosum* and *Bact. paratyphosum* A, and the same is true, if tubes are allowed to stand over night. *Bact. paratyphosum* B and the *Bact. dysenteriae* group show a weak reaction, when examined within three hours; but a stronger reaction if kept over night. *Bact. coli* shows weak formalin readings after three hours; but if kept over night, the result, with or without formalin, is the same.

For the graphical agglutination test, we must use non-spontaneous agglutination bacilli. However, by the addition of formalin, spontaneous agglutination is prevented while at the same time a specific agglutination regularly occurs. As shown in table 3 of the strains of spontaneous agglutination bacilli, *Bact. typhosum* and *Bact. paratyphosum* A yielded specially good results; *Bact. paratyphosum* B showed less influence, while *Bact. coli* was not influenced at all. When the tests were conducted without formalin, spontaneous and specific serum agglutinations usually present the same appearance microscopically and macroscopically, although certain strains of spontaneous agglutination bacilli sometimes yield weaker macroscopic reactions. It is evident that spontaneously agglutinating bacilli should not be used for graphical agglutination tests, and that formalin may be used to prevent the complex reaction.

TABLE 3

Comparative agglutination test, specific serum with 0.1 per cent formalin (final dilution) in 0.85 per cent salt solution and 0.85 per cent salt solution, have been used, with neutral broth cultures, 37°C.—twenty hours growth

Organism	Bact. typhosum							Bact. paratyphosum A							Bact. paratyphosum B							Bact. coli						Bact. dysenteriae.					
Number of strain	Specific serum	No. 1	No. 2	No. 3	Total 3	Nos. 1,2	Nos. 2,3	Total 2	Specific serum	No. 1	No. 2	No. 3	Total 3	Nos. 2,3	Nos. 3,2	Total 2	Specific serum	No. 1	No. 2	No. 3	Total 3	Nos. 2,3	Nos. 3,2	Total 2	Specific serum	No. 1	No. 2	No. 4	No. 3	Total 4	Specific serum	Shiga	Flexner	Strong	Hiss	Total
Spontaneous agglutination on culture		0	0	0	0	3	3	6		0	0	0	0	3	3	6		0	0	0	0	3	2	5		0	0	2	2	4		0	0	0	0	0
0.1 per cent formalin	1 to	5	5	5	15	5	5	10	1 to	5	5	5	15	4	4	8	1 to	5	5	4	14	5	5	10	1 to	5	5	5	5	20	1 to	5	5	4	5	19
Salt solution	25,000	4	4	4	12	5	4	9	10,000	4	3	3	10	3	3	6	1000	5	4	4	13	5	5	10	1500	5	5	5	5	20	2000	5	5	4	5	19
0.1 per cent formalin	1 to	2	3	2	7	2	1	3	1 to	2	1	2	5	2	1	3	1 to	1	2	1	4	2	1	3	1 to	3	3	3	3	12	1 to	4	3	2	2	11
Salt solution	100,000	1	2	1	4	3	2	5	50,000	1	0	0	1	2	1	3	5000	1	1	1	3	3	1	4	2000	3	3	3	3	12	10,000	4	3	2	1	10
Control:																																				
Formalin	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2	0	2	0	0	0	2	2	4	0	0	0	0	0	0
Salt solution	0	0	0	0	0	3	2	5	0	0	0	0	0	2	1	3	0	0	0	0	0	3	1	4	0	0	0	2	2	4	0	0	0	0	0	0

Key: 5, complete; 4, almost complete; 3, weak; 2, very weak; 1, trace; 0, negative agglutination.

ACTION OF FORMALIN ON PSEUDO AGGLUTINATION IN THE CROSS AGGLUTINATION REACTION WITH DIFFERENT IMMUNE SERA (TABLE 4)

In conducting cross agglutination tests with various immune sera pseudo agglutination usually did not take place with formalin. However, certain strains of bacilli showed marked agglutination, as well as specific agglutination, without formalin.

Bact. typhosum. Non-spontaneous agglutinating bacilli tested with the formalin showed very marked prevention of pseudo agglutination in these immune sera: *Bact. paratyphosum* A, and B, in horse serum, and even better in *Bact. dysenteriae* Shiga serum, but showed strong spontaneous agglutination without it.

The serum of polyvalent dysentery (1:2000), with *Bact. typhosum* had almost the same agglutinating reaction as the *Bact. dysenteriae* group, both with or without formalin. Hence, we may use the serum for identification of *Bact. typhosum* as well as for the *Bact. dysenteriae* group.

Bact. paratyphosum A. Formalinized *Bact. typhosum* serum (1:200), prevented pseudo agglutination entirely, with non-spontaneous agglutinating strains, while showing strong pseudo agglutination without formalin.

In the *Bact. paratyphosum* B serum (1:50), with non-spontaneous agglutinating bacilli, both with or without formalin, agglutination does not appear. This serum showed no tendency to cause pseudo agglutination for *Bact. paratyphosum* A.

Polyvalent dysentery serum, has agglutinated *Bact. paratyphosum* A, in almost the same degree with and without formalin; this serum seems to have a tendency to agglutinate this bacillus, but in this experiment the serum was highly concentrated 1:100. This reaction does not compare with the agglutinating reaction of *Bact. typhosum* or the *Bact. dysenteriae* group, when a dilution of 1:2000 or more is used.

Bact. dysenteriae Shiga serum (1:50), showed negative agglutination, both with and without formalin; this serum has no tendency to agglutinate with *Bact. paratyphosum* A.

Normal horse serum (1:50), shows very weak agglutination when using formalin, and stronger without it, but this serum shows a tendency to agglutinate only with *Bact. paratyphosum* A.

TABLE 4

Comparative study of pseudo agglutination with various sera using 20-hour cultures at 37°C., in 0.1 per cent formalinized salt solution (0.85 per cent) or normal salt solution (0.85 per cent) with neutral broth medium.

IMMUNE SERA FROM RABBIT OR SHEEP; TITRES OF SERUM	ORGANISM		BACT. TYPHOSUM				BACT. PARA A				BACT. PARA B				BACT. COLI				BACT. DYSENTERIAE					
	Number of strains		No. 1	No. 2	No. 3	Total 3	No. 1	No. 2	No. 4	Total 3	No. 1	No. 2	No. 3	Total 3	No. 1	No. 2	No. 3	Total 3	Shiga 1	Shiga T	Flexner	Hiss	Strong	Total 5
1:30,000	Bact. typhosum serum, 1:200	Formalin					0	0	0	0	3	0	0	3	0	0	0	0	0	0	0	0	0	0
		Salt solution					4	4	5	13	5	4	5	14	0	0	0	0	0	0	0	0	0	0
1:500	Bact. para. A serum, 1:50	Formalin	0	0	0	0					0	0	0	0	0	0	0	0	3	3	2	2	3	13
		Salt solution	4	3	4	11					4	4	4	12	0	0	0	0	3	1	1	1	1	7
1:1500	Bact. para. B serum, 1:50	Formalin	0	0	0	0	0	0	0	0					0	0	0	0	0	0	0	0	0	0
		Salt solution	0	3	2	5	0	0	0	0					0	0	0	0	0	0	0	0	0	0
1:4000	Polyval. dysentery serum, 1:100	Formalin	5	5	5	15	3	3	4	10	3	3	1	7	5	5	5	15	5	5	5	5	5	25
		Salt solution	5	5	5	15	4	4	4	12	4	5	4	13	5	5	5	15	5	5	5	5	5	25
1:3500	Dysentery Shiga serum, 1:50	Formalin	0	0	0	0	0	0	0	0	2	0	1	3	0	0	3	3			2	0	4	6
		Salt solution	3	0	1	4	0	0	0	0	3	3	3	9	1	2	3	6			1	0	3	4
1:1000	Normal horse serum, 1:50	Formalin	2	0	0	2	2	1	2	5	0	0	3	3	5	5	5	15	5	5	5	5	5	25
		Salt solution	4	3	2	9	3	1	3	7	5	3	4	12	5	5	5	15	5	5	5	5	5	25
Control		Formalin	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
		Salt solution	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

Key: 5, complete; 4, almost complete; 3, weak; 2, very weak; 1, trace; 0, negative agglutination.

Bact. paratyphosum B. Formalinized *Bact. typhosum* serum (1:200), practically did not show the pseudo agglutination; most cases were negative (with the exception of no. 1 strain, which had strong agglutination with this serum, and weak agglutination with homologous serum,—a peculiar strain but nevertheless typical *Bact. paratyphosum* B), while showing strong agglutination without formalin.

Bact. paratyphosum A serum (1:50); pseudo agglutination is shown to be negative with formalin, but strong agglutination occurs without formalin; hence, this serum does not have a tendency to pseudo agglutination with *Bact. paratyphosum* B, if used with formalin.

Polyvalent dysentery serum (1:100), or normal horse serum (1:50), shows negative or weak pseudo agglutination with formalin, and strong agglutination without it.

Bact. dysenteriae Shiga serum; many strains are negative or show a weak reaction with formalin, but show more pseudo agglutination without formalin. This, however, is a strongly concentrated serum (1:50), if it is diluted 1:100, it does not agglutinate with *Bact. paratyphosum* B.

Bact. coli. As a rule *Bact typhosum* serum (from immune rabbit or sheep), with or without formalin, shows no agglutination in high concentrations, as 1:200.

Bact. paratyphosum A and B serum (from immune rabbit), generally showed no agglutination with *Bact. coli.*

Polyvalent dysentery serum showed strong agglutination with *Bact. coli* either with or without formalin, almost equal to that of the *Bact. dysenteriae* group, as observed by Kligler. This serum we could use for a diagnostic test for *Bact. coli.*

Bact. dysenteriae Shiga serum (1:50), showed slight or negative agglutination with formalin, but more without formalin; it is however not a marked reaction.

Bact. dysenteriae group. With *Bact. typhosum* serum (1:200), and *Bact. paratyphosum* B serum (1:50), with or without formalin, pseudo agglutination was generally negative, but with *Bact. paratyphosum* A serum 1:50 (obtained from rabbit), there was slight agglutination.

Bact. dysenteriae Shiga serum (1:50), shows slight agglutination with the Flexner and Strong strains; but the reaction is stronger with formalin than without; with the Hiss strain, there is no agglutination either with or without formalin. This serum does not have a tendency to group agglutination as do sera of other *Bact. dysenteriae* groups, and horse serum serves for agglutination as well as specific serum.

HORSE SERUM AGGLUTINATION FOR BACT. DYSENTERIAE GROUP AND BACT. COLI. (TABLE 5)

Lentz, Park and Williams have found that *Bact. dysenteriae* is agglutinated by normal horse serum, and Gasiakoski, Park and Williams found that *Bact. coli* is agglutinated by normal horse serum. In my experiments nearly every strain of *Bact. dysenteriae* and *Bact. coli* was readily agglutinated by normal horse serum. All samples of horse serum employed, as shown in the table, produced agglutination of both groups of bacilli; only one strain of *Bact. dysenteriae* Shiga no. T obtained from Dr. Shiga, showed negative or weakly positive agglutination.

I have also found that the sera of horses immunized with different bacteria as for instance *Corynebact. diphtheriae*, the meningococcus, *Bact. paratyphosum* and the pneumococcus, have the same agglutinating power as normal horse serum.

COMPARATIVE AGGLUTINATION TESTS WITH 0.7 PER CENT ACID, NEUTRAL, AND 0.3 PER CENT ALKALINE BROTH CULTURE MEDIA (TABLE 6)

Bact. typhosum. Freshly prepared acid broth was used; a heavy growth and actively motile organisms with a tendency to spontaneous agglutination were obtained within 20 hours; with formalin, spontaneous agglutination was prevented almost entirely, while without formalin considerable spontaneous agglutination occurred. These cultures were more easily agglutinated with specific serum than cultures grown in neutral or alkaline broth. According to Dreyer, Eisenberg and Volk, Joos, and Weiss acid increases the agglutinating power.

TABLE 5

Agglutination of Bact. dysenteriae and Bact. coli, with normal and immunized horse serum with different organisms

Organism				Bact. dysenteriae						Bact. coli				
Number of strain				Shiga No. 1	Shiga No. T	Flexner No. 1	Flexner No. 2	Strong	Hiss	Number 1	Number 2	Number 4	Number 6	Number 7
Normal and Immunized sera of Horse	Normal horse	No. 2	1:200	5	1	5	5	4	5	5	5	5	5	3
			1:1000	5	0	2	2	0	1	3	3	5	2	1
		No. 3	1:200	5	3	3	3	1	4	4	5	1	3	3
			1:1000	5	1	1	1	0	1	1	2	0	0	0
	Anti-meningococcus	No. 6	1:200	5	2	5	4	2	5	5	5	5	5	4
			1:1000	5	0	1	0	0	1	5	4	3	2	1
		No. 7	1:200	5	0	5	5	5	5	5	5	5	5	4
			1:1000	5	0	4	2	5	2	3	1	3	2	1
	Anti-diphtheria	No. 8	1:200	5	0	5	5	4	5	5	5	5	2	4
			1:1000	5	0	2	1	1	5	5	2	3	0	1
		No. 9	1:200	5	0	5	5	5	5	5	4	5	3	4
			1:1000	5	0	3	1	2	3	3	2	2	1	1
	Anti-pneumococcus		1:200	5	0	5	5	5	5	5	4	5	4	4
			1:1000	5	0	3	2	1	4	4	1	3	1	1
	Anti-B. paratyphosum A		1:200	5	4	5	4	5	5	5	5	5	4	3
			1:1000	5	1	5	1	4	3	5	5	5	1	1
Control				0	0	0	0	0	0	0	0	0	0	0

Key: 5, complete; 4, almost complete; 3, weak; 2, very weak; 1, trace; 0, negative agglutination.

In neutral broth the motility, spontaneous agglutination and also specific serum agglutination, were much weaker than in acidified broth. Formalin, prevented spontaneous agglutination entirely.

In alkaline broth most strains of *Bact. typhosum* showed a much weaker growth, motility and spontaneous agglutination.

TABLE 6

Comparative agglutination tests with 0.7 per cent acid, neutral, and 0.3 per cent alkaline broth cultures, at 37°C.—twenty hours growth

THREE KINDS OF CULTURE MEDIA		+ 0.7	±	− 0.3	+ 0.7	±	− 0.3	+ 0.7	±	− 0.3	+ 0.7	±	0.3 l	+ 0.7	±	0.3 l	TOTAL + 0.7	TOTAL ±	TOTAL 0.3 l
Number of Bact. typhosum		No. 1			No. 2			No. 3			Nos. 1, 2			Nos. 29, 2			5 strains		
Motility		+	+	<+	+	+	?	+	<+	?	+	+	<+	+	+	<+			
Spontaneous agglutination		1	0	0	0	0	0	1	0	0	5	3	2	4	3	0	11	6	2
Dilution of serum	1:25,000	5	4	2	5	4	3	5	4	2	5	5	3	5	4	3	25	21	13
	1:100,000	3	1	0	3	2	1	3	2	1	3	2	1	3	2	1	15	9	4
Control		0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	1	0	0
Number of Bact. para A		No. 1			No. 2			No. 3			Nos. 2, 2			Nos. 17, 2			5 strains		
Motility		+	<+	<<+	+	+	<+	+	<+	<+	+	+	+	+	+	<+			
Spontaneous agglutination		2	0	0	0	0	0	0	0	0	3	2	1	3	2	0	8	4	1
Dilution of serum	1:10,000	5	5	4	5	4	3	5	4	3	5	4	3	5	4	1	25	21	14
	1:50,000	3	2	1	3	2	1	3	2	1	2	2	1	1	0	0	12	8	4
Control		0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Number of Bact. para B		No. 1			No. 2			No. 3			Nos. 1, 2			Nos. 7, 2			5 strains		
Motility		+	+	+	+	+	+	+	+	+	+	+	+	+	+	+			
Spontaneous agglutination		1	0	0	0	0	0	0	0	0	4	3	0	3	3	3	8	6	3
Dilution of serum	1:1000	5	4	4	5	5	5	4	4	3	4	3	3	5	4	4	23	20	19
	1:5000	2	2	2	3	3	3	2	2	2	2	2	2	3	3	2	12	12	11
Control		0	0	0	0	0	0	0	0	0	0	0	0	1	1	1	1	1	1

Bact. dysenteriae		Shiga 1			Shiga T			Flexner			Hiss 1			Strong			5 strains		
Spontaneous agglutination		0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Dilution of serum	1:5000	5	5	4	5	5	5	4	3	3	5	5	5	5	4	2	24	22	19
	1:15,000	4	4	3	2	2	2	2	1	0	3	3	3	2	1	0	13	11	8
Control		0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Number of Bact. coli		No. 1			No. 2			No. 4			Nos. 3, 2			Nos. 15, 2			5 strains		
Motility		0	0	0	0	0	0	0	0	0	0	0	0	<+	<+	<+			
Spontaneous agglutination		0	0	0	0	0	0	0	0	0	2	1	1	2	2	2	4	3	3
Dilution of serum	1:500	5	5	5	5	5	5	5	5	4	5	5	5	5	5	5	25	25	24
	1:1500	3	3	3	3	3	3	3	3	2	4	4	4	4	4	4	17	17	16
Control		0	0	0	0	0	0	0	0	0	2	2	2	1	1	1	3	3	3

Key: +, active; <+, usual; <<+, slow motility; 5, complete; 4, almost complete; 3, weak; 2, very weak; 1, trace; 0, negative agglutination.

The specific serum agglutination in this medium was also weak. Therefore the use of alkaline media for *Bact. typhosum* is not favorable for agglutination tests. According to Park and Williams, and Tarchitte, the agglutinating power of the organism is lost in an alkaline medium.

Bact. paratyphosum A. These cultures were grown in acid, neutral and in alkaline broth, the reactions of the media being the same as with *Bact. typhosum.*

Bact. paratyphosum B. In general the differences in growth and motility in acid, neutral and in alkaline broth were far less marked than in the case of *Bact. typhosum* and *Bact. paratyphosum* A. Both spontaneous and specific agglutination were strongest in the acid medium, but the differences were less than with *Bact. typhosum* or *Bact. paratyphosum* A. However, there is some variation depending upon each strain.

Bact. dysenteriae and Bact. coli group. On an average the specific serum agglutination test was slightly stronger in acid broth; otherwise the results were almost the same for acid, neutral, and alkaline broth cultures.

In the preceding experiments the nutrient broth was prepared and titrated with phenolphthalein, just before using, as I have reported. The medium becomes more acid on standing, due to the absorption of carbon dioxide from the atmosphere.

COMPARATIVE AGGLUTINATION TEST WITH AGAR CULTURE EMULSIFIED IN SALT SOLUTION OR BROTH MEDIUM

Bass and Watkins, Buxton and Vaugham, Kolle, Jordan, Park and Williams, Ritchie, Weil, Wretoski and other workers have used salt solution, for emulsifying the agar culture for the Widal test.

Block, Grumbaum, Durham, and Ker used broth medium with the agar cultures; Hiss and Zinsser used agar cultures emulsified in salt solution or broth medium.

In my experiments, I have not found any particular difference between these fluids. But agglutination is slightly stronger with broth emulsions of *Bact. typhosum*, *Bact. paratyphosum* A, *Bact. dysenteriae* and *Bact. coli*; with *Bact. paratyphosum* B the reactions are about equal.

There is slightly less tendency to spontaneous agglutination in a broth medium than in salt solution; therefore, I have concluded that salt is better for preparing suspensions with agar cultures for Widal test.

EFFECT OF SODIUM CHLORID ON AGGLUTINATION TESTS (TABLE 7)

Malvoz used agar cultures emulsified with distilled water to avoid chemical changes resulting from sodium chlorid. Weil, working with agar cultures, obtained the same agglutinating reaction, when using either distilled water, or 0.85 per cent sodium chlorid. Asakawa, Bordet, Chick, Joos, Jordan, and Porges claim that sodium chlorid is necessary for the agglutination test. Dreyer, Krumbaar and Smith used tap water in diluting the serum for broth cultures and the results were better than with sodium chlorid. Chick claims 0.42 per cent sodium chlorid gave good results.

In my observation 24 hour agar cultures of *Bact. typhosum*, *Bact. paratyphosum* A and B, *Bact. dysenteriae and Bact. coli* emulsified with distilled water were agglutinated by specific serum in dilutions of 1:40 or 1:50, as well as when emulsified with 0.85 per cent salt solution; in this case a certain amount of salt contained in the serum aided agglutination. In weak dilutions of 1:80 or more, there was no agglutination in distilled water (with one exception), but marked agglutination in salt solution. Evidently at that dilution, the salt content of the serum was not sufficient. Only one strain of *Bact. dysenteriae* Flexner no. 2, showed constant agglutination in distilled water with a specific serum dilution of 1:80.

Spontaneous agglutination appeared with spontaneous agglutinating bacilli in almost every instance, when using either distilled water or salt solution.

Quantity of sodium chlorid. I have tried various dilutions of salt, varying from 0.05 to 5 per cent, with agar cultures, but no apparent differences were observed in their effect on the agglutination reaction.

It is my conclusion, that only a trace of sodium chlorid is necessary for the Widal test. Since strong solutions are of no advantage, I believe ordinary physiological 0.85 per cent salt is

TABLE 7

Comparative agglutination tests of agar cultures (37°C.—twenty-four hours), emulsified with 0.85 per cent salt solution and distilled water

Organism			BACT. TYPHOSUM				BACT. PARA A				BACT. PARA B				BACT. COLI				BACT. DYSENTERIAE				
Number of strain			No. 1	No. 2	Nos. 1, 2	No. 7	No. 1	No. 2	Nos. 2, 2	Nos. 4, 2	No. 1	No. 2	Nos. 2, 2	Nos. 4, 2	No. 4	No. 6	Nos. 1, 2	Nos. 2, 2	Shiga T	Flexner 1	Flexner 2	Strong	Hiss
Spontaneous agglutination on cultures			0	0	2	2	0	0	2	2	0	0	2	3	0	0	2	3	0	0	0	0	0
Dilution of specific serum	1:40	Water	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5
		Salt solution	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5
	1:80	Water	0	0	1	1	0	0	2	2	0	0	1	1	0	0	3	3	0	0	5	0	0
		Salt solution	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5
Control		Water	0	0	1	1	0	0	1	1	0	0	1	1	0	0	1	3	0	0	0	0	0
		Salt solution	0	0	1	1	0	0	1	1	0	0	1	1	0	0	1	3	0	0	0	0	0

Key: 5, complete; 4, almost complete; 3, weak; 2, very weak; 1, trace; 0, negative agglutination.

quite as satisfactory for agar or broth cultures, especially as it is to be found in most laboratories.

There were no indications that the use of tap water or distilled water was better than salt solution, when we used broth cultures.

COMPARATIVE AGGLUTINATION TESTS WITH VARIOUS CULTURE MEDIA (TABLE 8)

In conducting agglutination tests of the colon-typhoid group various kinds of culture media have been used. The cultures were grown at 37°C. for 24 hours in neutral broth, one per cent glucose broth, one per cent pepton water and on agar, the agar cultures being emulsified with salt solution.

Bact. typhosum. Grown in plain broth, glucose broth, pepton water and on agar, it showed the strongest agglutination with specific serum in the first named medium, becoming weaker in each of the others in the order given; the reaction was very weak with agar cultures. Dreyer claims that glucose broth cultures have a tendency to spontaneous agglutination, but my results in using glucose broth were quite irregular; spontaneous agglutination occurred in some cases, while in others there was no spontaneous agglutination, but there was generally negative or weak spontaneous agglutination with both spontaneous and non-spontaneous agglutinating types.

As compared with cultures in plain broth those grown in glucose broth showed very irregular and weak motility. Formalinized serum showed stronger agglutination than plain serum; especially marked was the difference with glucose broth. With plain broth or pepton water or agar cultures, the difference was a great deal less marked.

Bact. paratyphosum A. Cultures grown in glucose broth showed weak motility, many strains agglutinating spontaneously, even though non-spontaneous agglutinating bacilli were used. Formalin does not prevent this agglutination.

In specific agglutination tests glucose broth cultures showed considerable reaction, even more than with pepton water or agar cultures. However, these results are due to the spontaneous agglutination in the glucose broth. It is evident, that this

TABLE 8

Comparative agglutination tests, with neutral broth, 1 per cent glucose broth, 1 per cent pepton water, and agar (in salt solution) 37°C.—twenty-four hours culture, Added O, 1 per cent formalin

Culture media	Plain broth	1 per cent glucose broth	1 per cent pepton water	Agar	Plain broth	1 per cent glucose broth	1 per cent pepton water	Agar	Plain broth	1 per cent glucose broth	1 per cent pepton water	Agar	Plain broth	1 per cent glucose broth	1 per cent pepton water	Agar	TOTAL: Plain broth	TOTAL: 1 per cent glucose broth	TOTAL: 1 per cent pepton water	TOTAL: Agar
Bact. typhosum	No. 1				No. 2				No. 3				No. 6				4 strains			
Motility	+	<+	+	+	+	<+	+	+	+	+	+	+	+	<+	+	+				
Spontaneous agglutination on culture	0	0	0	0	0	1	1	0	0	0	0	0	0	0	1	0	0	1	2	0
Bact. typhosum serum 1:25,000	5	4	3	2	5	4	4	2	5	4	3	2	5	4	4	2	20	16	14	8
Bact. typhosum serum 1:100,000	3	2	1	0	2	2	1	0	3	2	0	0	3	2	2	1	11	6	4	1
Control	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Bact. para A	No. 1				No. 2				No. 3				No. 4				4 strains			
Motility	+	<+	+	<+	<+	<+	<+	<+	+	+	+	<+	+	+	+	<+				
Spontaneous agglutination on culture	0	2	2	0	0	1	0	0	0	3	0	0	0	0	0	0	0	6	2	0
Bact. para A serum 1:10,000	5	5	4	2	4	4	4	2	5	5	4	2	5	5	4	4	19	19	16	10
Bact. para A serum 1:50,000	2	3	2	0	1	1	0	0	1	3	1	0	2	1	1	0	6	8	4	0
Control	0	2	1	0	0	0	0	0	0	3	0	0	0	0	0	0	0	5	1	0

Bact. para B.	No. 1				No. 2				No. 3				No. 4				4 strains			
Motility	+	<+	+	+	+	<+	<+	<+	+	<+	+	+	+	<+	+	<<+				
Spontaneous agglutination on culture	0	2	1	0	0	1	1	1	0	1	0	1	0	3	2	0	0	7	4	2
Bact. para B serum 1:2,000	3	4	3	3	4	4	5	3	3	3	3	2	4	4	4	3	14	15	15	11
Bact. para B serum 1:5,000	2	3	2	2	2	3	3	1	2	2	1	0	2	4	3	2	8	12	9	5
Control	0	3	0	0	0	2	1	0	0	1	0	0	0	2	1	0	0	8	2	0
Bact. coli	No. 1				No. 2				No. 4				No. 6				4 strains			
Motility	<+	<+	<+	<+	0	0	0	0	0	0	0	0	<+	<+	<+	<+				
Spontaneous agglutination on culture	0	2	1	0	0	2	1	1	0	2	1	0	0	2	0	0	0	8	3	1
Horse serum 1:700	5	5	4	2	5	5	4	2	5	5	5	3	3	3	2	2	18	18	15	9
Horse serum 1:30,00	2	3	2	2	2	3	4	2	2	2	2	1	0	1	1	0	6	9	9	5
Control	0	2	0	0	0	3	3	0	0	2	0	0	0	1	1	0	0	8	4	0
Bact. dysenteriae	Shiga No. 1				Flexner				Hiss				Strong				4 strains			
Spontaneous agglutination on culture	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	
Polyv. B. dysenteriae serum 1:2,000	5	5	5	5	5	3	4	4	5	4	4	4	5	3	5	4	20	15	18	17
Polyv. B. dysenteriae serum 1:15,000	4	3	4	4	2	0	2	1	2	1	2	1	2	0	2	2	10	4	10	8
Control	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

Key: +, active; <+, slow; <<+, very slow motility; 5, complete; 4, almost complete; 3, weak; 2, very weak; 1 trace; 0, negative agglutination.

medium is not suitable for the agglutination test for *Bact. paratyphosum* A.

Occasionally spontaneous agglutination in pepton water cultures is difficult to prevent, while in plain broth it is relatively easy to prevent; in pepton water there is but a slight tendency to spontaneous agglutination even with non-spontaneous agglutinating bacilli. Specific agglutinations with pepton water cultures were slightly weaker than with plain or glucose broth cultures. With formalin the reaction was slightly stronger than without.

With agar cultures the agglutination reaction was generally slow and weak and appeared somewhat incomplete, depending however upon the strain and age of the culture. With or without formalin almost the same results are obtained with non-spontaneous agglutinating bacilli.

Bact. paratyphosum B. Glucose broth cultures of *Bact. paratyphosum* B usually show weak motility and strong spontaneous agglutination, which is not prevented when treated with formalin; even non-spontaneous agglutinating bacilli give the same result. This medium does not appear to be satisfactory for the Widal test with *Bact. paratyphosum* B.

Cultures in plain broth and pepton water showed more vigorous motility and almost the same agglutinating reaction with specific serum. But in pepton water with non-spontaneous agglutinating bacilli, there was a slight tendency to spontaneous agglutination even when the cultures were mixed with salt solution or with formalin. It appears that plain broth cultures are more reliable than are those in pepton water.

The agar cultures showed weak specific agglutination in comparison with the other three kinds of media, but did not have the tendency to spontaneous agglutination.

Bact. coli. Glucose broth cultures of most strains of *Bact. coli* grown for eighteen to twenty-four hours at 37°C. showed considerable spontaneous agglutination with both spontaneous and non-spontaneous agglutinating bacilli; hence this medium cannot be used for the specific agglutination test.

Certain strains in pepton water cultures showed a slight tendency to spontaneous agglutination, while in broth they showed no spontaneous agglutination. The specific agglutinations were weaker in pepton water than broth medium; therefore I consider the latter most suitable for the Widal test with *Bact. coli.*

Agar cultures show very weak agglutination, but there is no tendency to spontaneous agglutination with *Bact. coli.*

Bact. dysenteriae group. This group showed strong specific agglutination in plain broth cultures, it was slightly weaker in pepton water and agar and especially weak in glucose broth; but glucose broth did not show spontaneous agglutination.

TABLE 9

Relation of age to specific agglutinating power with non-spontaneous agglutination bacilli, grown on broth culture medium

Organism		Bact. typhosum				Bact. para A				Bact. dysenteriae					
Number of strain		No. 1	No. 2	No. 6	Total 3	No. 1	No. 2	No. 3	Total 3	Shiga No. 1	Flexner	Strong	Hiss	Total 4	Shiga No. T
Ages of cultures and specific serum	1 day	5	5	5	15	5	5	5	15	5	5	5	5	20	2
	2 days	5	4	5	14	5	4	4	13	5	5	5	3	18	3
	3 days	4	3	4	11	4	3	3	10	4	4	4	2	14	4
	5 days	3	2	3	8	3	2	2	7	3	3	2	1	9	5

Key: 5, complete; 4, almost complete; 3, weak; 2, very weak; 1, trace; 0, negative agglutination.

RELATION OF AGGLUTINATION POWER TO AGE OF CULTURES (TABLE 9)

Dreyer claims that cultures more than one day old had a weak agglutinating power with specific serum. My experiments for a period of five days, with *Bact. typhosum*, *Bact. paratyphosum* A and the *Bact. dysenteriae* group, indicated that cultures on neutral broth medium one day old were agglutinated best, the reactions become weaker each day thereafter. However, I found that one strain of *Bact. dysenteriae* Shiga no. T showed just the opposite result, the agglutinating power becoming stronger with older cultures over a period of five days.

SPONTANEOUS AGGLUTINATION ACCORDING TO AGE OF CULTURES (TABLE 10)

When spontaneous agglutinating bacilli were grown in neutral broth or pepton water, spontaneous agglutination was more vigorous when cultures were but one day old, diminishing steadily afterwards and almost disappearing on the fifth day.

I concluded therefore that both spontaneous and specific agglutinating power diminsh with the age of most strains of the colon-typhoid group.

TABLE 10

Spontaneous agglutination according to age of bacilli growing in plain broth at 37° C.

Organism		Bact. typhosum			Bact. para A			Bact. para B			Total
Number of strain		No. 7	No. R	No. 18	No. 1	No. 2	No. 6	No. 1	No. 2	No. 3	9
Ages of cultures	1 day	3	3	3	3	2	3	3	3	3	26
	2 days	2	2	2	2	2	2	2	3	2	19
	3 days	1	1	2	1	1	1	1	2	1	11
	5 days	0	0	1	0	0	0	0	1	0	2

Key: 3, strong; 2, medium; 1, weak spontaneous agglutination.

COMPARATIVE RESULTS OF MICROSCOPIC AND MACROSCOPIC AGGLUTINATION TESTS

Chick, Dreyer, Garrow, Jordan, Panton, Walker, Wilson, and several other investigators prefer the macroscopic method; Delepine, Ritchie, and several other workers have used the microscopic method.

In using non-spontaneous agglutinating cultures, strong agglutination could be seen equally well with either method, and weak agglutination better by the microscopic method. For instance, in some cases which appeared negative macroscopically, small clumps could be seen microscopically when examined at the end of three hours. These small clumps are of great value in certain cases of very weak reactions. This is especially noticeable with the *Bact. dysenteriae* group, but less so for

Bact. typhosum, *Bact. paratyphosum* A and B, and the *Bact. coli* group.

In the case of *Bact. typhosum* and *Bact. paratyphosum* A and B, spontaneous agglutination generally appears weak macroscopically and strong microscopically; but with *Bact. coli* it appears to be almost the same with either method. It is specially to be noted that when using spontaneous agglutinating bacilli for the Widal test, the macroscopic method was found more reliable.

For the fundamental or graphic agglutination test, I used non-spontaneous agglutinating bacilli. When agglutination was

TABLE 11

Agglutination tests at different temperatures

Organism		Bact. typhosum				Bact. para A				Bact. para B				Bact. coli				Bact. dysenteriae			
Number of strain		No. 1	No. 2	No. 3	Total 3	No. 1	No. 2	No. 3	Total 3	No. 1	No. 2	No. 3	Total 3	No. 1	No. 2	No. 3	Total 3	Shiga	Flexner	Hiss	Total 3
With each specific serum	Room temperature	5	5	5	15	5	5	5	15	5	5	5	15	5	5	5	15	5	5	5	15
	37°C. water bath	5	5	5	15	5	5	5	15	5	5	5	15	5	5	5	15	5	5	5	15
	45°C. water bath	4	4	4	12	4	4	4	12	4	4	4	12	4	4	4	12	4	4	4	12
	55°C. water bath	3	3	2	8	4	4	3	11	3	3	3	9	4	3	3	10	3	3	3	9
Controls of all		0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

Key: 5, complete; 4, almost complete; 3, weak; 2, very weak; 1, trace; 0, negative agglutination.

weak or the case questionable, I resorted to the microscopic method for final determination.

AGGLUTINATION AT DIFFERENT TEMPERATURES (TABLE 11)

Delepine, Jordan, Konrich, Lion, Meyer and Kilgore, Widal, and many others conducted agglutination tests at 37°C.; Dreyer and Blake, Hetsch, Kolle, Kutscher, Porges, and Weil advise the use of 50°C. to 55°C.; Joos 35°C. to 40°C.; Berliner and Cohn, and Durham recommend room temperature.

Recently many workers have read the test, after keeping the tubes in the water bath two to three hours at from 50°C. to 55°C. then leaving them at room temperature or in the cold room over night.

In my observations on the effect of temperature of the agglutination with *Bact. typhosum*, *Bact. paratyphosum* A and B, *Bact. coli*, and the dysentery group, the tubes kept in a water bath for two hours at 45°C., showed a slightly weaker reaction than those kept at room temperature or 37°C., while at 55°C., the reaction was much weaker than at 45°C. The results, after leaving the tubes for 2 to 3 hours, or even for twenty-four hours at room temperature, were just the same as they were when the tubes were placed in the water bath at 37°C. for two to three hours and then left standing at room temperature over night.

TABLE 12

Agglutination tests conducted with bacilli after heating at 55°C. for two hours

Organism		Bact. typhosum				Bact. para A				Bact. para B				Bact. coli				Bact. dysenteriae			
Number of strain		No. 1	No. 2	No. 3	Total 3	No. 1	No. 2	No. 3	Total 3	No. 1	No. 2	No. 3	Total 3	No. 1	No. 2	No. 3	Total 3	Shiga	Flexner	Hiss	Total 3
With specific serum	Unheated	5	5	5	15	5	5	5	15	5	5	5	15	5	5	5	15	5	5	5	15
	55°C. 2 hours	3	3	3	9	3	2	2	7	3	4	3	10	3	4	3	10	2	3	2	7
Control of both		0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

Key: 5, complete; 4, almost complete; 3, weak; 2, very weak; 0, negative agglutination.

In the next experiments (table 12), bacilli were heated at 55°C. for two hours before conducting the agglutination test; it became evident that these bacilli had less agglutinating power than the unheated bacilli. On the other hand, the serum which had been heated at 55°C. for two hours gave just the same results as serum which had not been heated.

When temperatures from 45°C. to 55°C. were used, the clumps were smaller than when the test was conducted at room temperature or 37°C. This was true when either the microscopic or macroscopic methods were employed.

The latter temperature is therefore to be preferred, with the colon-typhoid group with or without formalin; the end results, in relation to the temperature, are just the same.

THE TIME FACTOR IN THE AGGLUTINATION TEST

Agglutination tests after various periods of time were carried out with the colon-typhoid group, in relation to certain titres of the serum.

Bact. typhosum showed the reaction quicker than the other organisms, but the agglutination reaction of most of the strains was completed only after standing over night, instead of after three hours. However, some strains showed almost complete agglutination in three hours; others only a trace in this time. Of course with strong serum, agglutination took place soon after mixing with the bacterial suspension.

With *Bact. paratyphosum* B, the results were of the same order, but less rapid than with *Bact. typhosum.*

Bact. coli comes after *Bact. paratyphosum* B, with regard to the speed of the reaction.

Bact. paratyphosum A, and *Bact. dysenteriae* show relatively very slow agglutination; in most instances, the reaction was definitely obtained only after standing over night.

However, the speed of the reaction varies with the strains of bacilli; some strains were always quickly agglutinated and others more slowly.

THE USE OF SEVERAL STRAINS FOR THE WIDAL TEST (TABLE 13)

In the Widal test most bacteriologists use only one strain of bacillus with the serum of the patient. This method does not give reliable results. Ordinarily we use polyvalent immune serum for the identification of a bacillus; for the same reason we should use several strains in testing the serum of a patient.

A serum agglutination reaction depends upon the organism infecting the patient. If the serum agglutinates the stock culture, it indicates a close relationship between this organism and the organism which infected the patient; a negative test indicates distant or no relationship.

In a case of typhoid fever (P. B. G. Hospital) the serum showed positive agglutination for three strains (nos. 1, 4, 33), but negative for three others (nos. 3, 7, 29), and doubtful for one more (no. 2).

In performing this test, therefore, I have found it convenient to grow each strain (6 or more) of this organism in broth medium at 37°C., for eighteen to twenty hours. Formalin 0.1 per cent is added to each of the cultures, which are then all mixed together, and are ready for immediate use; or they may be stored in a cold room until needed, as in Dreyer's method. This preparation gives reliable results in diagnosis with an unknown serum.

TABLE 13

The serum of typhoid fever, Widal test with different strains

		BACT. TYPHOSUM 7 STRAINS						
		No. 1	No. 4	No. 33	No. 2	No. 3	No. 7	No. 29
Serum dilutions of typhoid fever	1:25	5	5	5	3	1	0	3
	1:50	5	4	4	2	0	0	1
	1:100	4	3	3	1	0	0	0
	1:150	3	2	1	0	0	0	0
	1:200	1	0	0	0	0	0	0
Results		+	+	+	?	0	0	0

Key: 5, complete; 4, almost complete; 3, weak; 2, very weak; 1, trace; 0, negative agglutination; +, positive reaction.

SUMMARY

1. Formalin 0.05 to 0.2 per cent prevents spontaneous agglutination almost entirely in some instances and entirely in others with *Bact. typhosum* and *Bact. paratyphosum* A and in a lesser degree with *Bact. paratyphosum* B; with *Bact. coli* formalin has no effect in this regard.

2. One-tenth per cent formalin prevents spontaneous agglutination and increases specific agglutination, having a strong effect on *Bact. typhosum* and *Bact. paratyphosum* A, but less on *Bact. paratyphosum* B and *Bact. dysenteriae* and none on *Bact. coli.*

3. Formalin prevents pseudo agglutination to a great extent in cross agglutination test with different sera.

4. Cultures in acid medium in which there is heavy growth and active motility, yield stronger agglutination than cultures

in neutral broth with *Bact. typhosum* and *Bact. paratyphosum* A; this effect is less noticeable in cultures of *Bact. paratyphosum* B, *Bact. dysenteriae* and *Bact. coli*, but the agglutinating power of these organisms is very weak in alkaline media. Alkaline media are therefore not suitable for the agglutination test with cultures of these bacteria.

5. Agar cultures emulsified with 0.85 per cent salt solution or neutral broth are almost equal in susceptibility to agglutination, but certain strains have a tendency to spontaneous agglutination in broth medium; therefore salt solution is better.

6. As to the effect of sodium chlorid on agglutination reactions I could find no difference in the degree of agglutination reaction when using strong or weak solutions of sodium chlorid; only traces are necessary for agglutination.

7. Glucose broth cultures of *Bact. paratyphosum* A and B and *Bact. coli* showed a tendency to spontaneous agglutination; *Bact. typhosum* showed weaker specific agglutination in glucose broth, but stronger than in pepton water, only occasionally showing spontaneous agglutination. The dysentery group yielded weaker agglutination with glucose broth cultures than with plain broth, pepton water and agar cultures.

Pepton water cultures in general show a slight tendency to spontaneous agglutination, when non-spontaneous agglutinating bacilli are grown therein. This spontaneous agglutination is difficult to prevent even when formalin is used and the specific agglutination reactions of *Bact. typhosum*, *Bact. paratyphosum* A and B and *Bact. coli* are weaker than in plain broth or glucose broth. On the other hand the dysentery group yielded stronger specific agglutination reactions in this medium than in glucose broth.

Agar cultures showed much weaker agglutination than broth and pepton water cultures.

In general, plain broth cultures gave the best results.

8. Susceptibility of most cultures to agglutination decreases with age; eighteen to twenty-four hours cultures were found best suited for agglutination tests.

9. The microscopic is more reliable than the macroscopic method for weak or graphical agglutination work; with strongly agglutinating sera showing the same reactions in both, the macroscopic method is preferred, because it is easier and quicker than the microscopic method. Therefore the macroscopic method is to be regarded as most satisfactory for usual work and particularly when many reactions are to be performed.

10. Weak agglutination results if bacilli are heated at a temperature of 45 to 55°C. for two to three hours; this temperature does not affect the agglutinating activity of serum. Room temperature and 37°C., both gave better results than higher temperatures for the incubation of tests.

11. Two to three hours are not enough for complete agglutination; the tests should be set aside and read the next day.

12. Normal horse serum showed strong agglutination reaction with *Bact. dysenteriae, Bact. coli* and other bacilli of the typhoid-colon group.

Polyvalent *Bact. dysenteriae* serum had strong agglutinating power for *Bact. typhosum* and *Bact. coli* as well as for the dysentery group.

13. In serum diagnosis with the Widal test, many different strains should be used since from one strain alone, reliable results cannot be obtained.

SUPPLEMENTARY NOTES

a. Eighteen to twenty-four hours cultures of the majority of strains of *Bact. coli* grown at 37°C., show a tendency to spontaneous agglutination in most culture media; cultures grown at room temperature show less and are therefore preferable.

b. Old stock cultures transplanted to a fresh culture medium at first show weak, or no, susceptibility to agglutination; however, after a few sub-cultures (once or twice a day, if actively growing bacilli), they regain this property. The same holds good for all cultures of new bacilli, and it is necessary therefore, for accurate agglutination work to test the agglutinability of the bacterial culture with a control serum of known agglutinating powers.

c. Most cultures grown at 37°C. for eighteen to twenty-four hours show a slight pellicle on the surface; to avoid mistaking this for spontaneous agglutination the cultures should be shaken and left at room temperature for a while, after the pellicle falls to the bottom of the test tube.

d. Heavy growths yield better results in the Widal test when diluted 2 or 4 times with salt solution.

e. Acid broth (0.2 per cent) cultures should be used for routine agglutination tests inasmuch as acidity is useful and greatly helps in weak agglutination.

f. It is well known that commercial formalin contains formic acid. Fearing that the formic acid would interfere with the Widal reaction, I have tried certain strains of *Bact. typhosum* and *Bact. paratyphosum* group with various dilutions of formic acid (0.1 to 10 per cent) and found that with these dilutions weak chemical agglutinations are produced, but the specific agglutination is diminished. With a weak solution of formalin (0.1 to 1 per cent) no chemical agglutination is produced and spontaneous agglutination is prevented, as above stated. Evidently the quantity of formic acid in these concentrations of formalin is so small that it does not interfere with the reaction. Commercial formalin gave satisfactory results throughout this work.

g. The addition of phenol (0.1 per cent) or corrosive sublimate (0.01 per cent) did not change normal agglutination power. Tricresol (0.1 per cent) slightly weakened agglutination in the colon-typhoid group. All substances produced weak or negative agglutination reactions when used in more concentrated solutions than those above mentioned.

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A STUDY OF SPONTANEOUS AGGLUTINATION IN THE COLON-TYPHOID GROUP OF BACILLI

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It is well recognized that spontaneous agglutination may occur in broth cultures of *Bact. typhosum* and *Bact. paratyphosum* A and B. Because of this phenomenon, the results of agglutination tests with specific sera are not reliable under such conditions, since it is practically impossible to distingush between spontaneous and specific agglutination.

Block in 1897 found that cultures which were transplanted too frequently, may agglutinate spontaneously. He further observed that when cultures were grown in alkaline broth the same phenomenon occurred. Delepine and Fison (1897) also noted spontaneous agglutination in cultures of *Bact. typhosum*. Kruse, Rittershaus, Kemp and Metz described spontaneous agglutination of typhoid and dysentery bacilli in plain broth cultures and in very concentrated peptone broth cultures. Nicolle (1898) reported that changes in the bacterial protein occurred in old cultures, and that the organisms became more sensitive and readily agglutinated spontaneously in such cultures. Smith and Reagh (1903) reported that the *Bact. icteroides* developed granular colonies on gelatin cultures; i.e. were agglutinated spontaneously. Steinhardt (1904) found that in agar cultures, showing spontaneous agglutination, the colonies were irregular in shape and less translucent than the colonies of the type which did not agglutinate spontaneously. Teague and McWilliams (1917) reported that organisms isolated from the blood of a rabbit which had been injected with *Bact. typhosum*,

produced different kinds of colonies when grown on agar plates, some being opaque and others transparent; in addition there were also differences in their contours, some having irregular outlines while others were round with smooth edges. Furthermore some colonies were small and others large.

APPEARANCE OF SPONTANEOUSLY AGGLUTINATED COLONIES ON AGAR PLATES

Plating many stock cultures upon two per cent agar plates we have found two distinct types of colonies. The first of these presents a smooth surface and a regular outline, and is not spontaneously agglutinated. The other type shows a much heavier growth, has a rough surface, is irregularly round, with a serrate border and is much more transparent, both at its center and border, than the first type of colony. That most of the colonies of this second type undergo spontaneous agglutination, can be noted by means of the low power microscope or hand lens. In some instances, upon sub-culturing, certain strains of those colonies which previously had been transparent became opaque and vice versa; or an organism originally producing small colonies produced large ones. These points of differentiation are not so important as those relating to the outline of the colony and smoothness or roughness of the surface. The available space for growth on the plate as well as the rate of growth, appear to influence these characteristics.

SPONTANEOUS AGGLUTINATION IN BROTH CULTURES (TABLE 1)

Steinhardt, Teague and McWilliams found in broth cultures of *Bact. typhosum*, which underwent spontaneous agglutination, flocculi or pellicle formation as contrasted with a uniform, cloudy growth of other organisms. Smith and Reagh showed that a broth culture of *Bact. icteroides* became clear after spontaneous agglutination had occurred.

We have observed spontaneous agglutination of *Bact. typhosum*, *Bact. paratyposum* A and B, *Bact. enteritidis*, *Bact. dysenteriae* and *Bact. coli* in pepton water, glycerol broth and glucose

broth. In many cases the broth was clear macroscopically, but the clumps were visible when a hanging drop was examined. More or less of the precipitate collected in the bottom of the tubes. In some instances flocculi and a pellicle were visible on the surface of the culture, and there was always a precipitation in the bottom, either spontaneous or following agitation of the tube. In other cases, small clumps could be seen with the naked eye scattered throughout the culture. With the typhoid and paratyphoid bacilli there was a uniform cloudy growth and apparently an absence of spontaneous agglutination when

TABLE 1

Variations of growth in broth medium. Cultures spontaneously agglutinated show clear supernatant fluid, precipitation, pellicle, flocculi or small clumps, others appear uniformly cloudy, to the naked eye like cultures not spontaneously agglutinated

ORGANISMS GROWN IN BROTH	PRECIPITATION, PELLICLE AND FLOCCULI	UNIFORMLY CLOUDY	TOTAL NUMBER OF STRAINS
Bact. typhosum	4	12	16
Bact. paratyphosum A	7	9	16
Bact. paratyphosum B	15	1	16
Bact. coli	5	0	5

A tabulation of our experiments shows the following results: *Bact. paratyphosum* B showed precipitation with formation of pellicles and small clumps in clear fluid in all cases with the exception of one strain. *Bact. coli*, 5 strains exhibited this same phenomenon in every case. *Bact. paratyphosum* A showed 7 positive and 9 negative, *Bact. typhosum* 4 positive and 12 negative tests.

examined with the naked eye, but with a hand lens, flocculi were clearly visible in the supernatant fluid.

We have, therefore, been led to conclude that in broth cultures when flocculi are visible, macroscopic observation may be relied upon; but in cloudy cultures, microscopic examination should always be employed.

In twenty-four hour cultures at 37°C., of microörganisms which do not undergo spontaneous agglutination, the growth in pepton water, glycerol or glucose broth, whether the reaction of the media be acid, neutral or alkaline, is generally uniformly cloudy. In some cases, however, there is a pellicle and flocculi are seen near the surface of such cultures.

Microscopic examination of the twenty-four-hour broth cultures by the hanging drop method showed differences in morphology and motility between those organisms which agglutinated spontaneously and those which did not. In most cases the bacilli which undergo spontaneous agglutination are longer, sometimes having the appearance of being fungiform and showing relatively greater motility. The bacilli which do not agglutinate spontaneously are shorter and are less motile.

COLONY CHANGES IN ARTIFICIAL CULTURE MEDIA (TABLE 2)

Löffler (1906) found four types of colonies of *Bact. coli:* (1) transparent, (2) flat, (3) thick, and (4) opaque. According to his evidence, these types are constant and do not change. Baerthlein (1911) reported a cholera vibrio colony that did not change after passage through animals, but in a stock culture twenty-two days afterwards, in several subcultures, the yellow type turned light and twenty-eight days later the light colony changed to a yellow one. Steinhardt found spontaneous agglutination in *Bact. typhosum* after the twentieth passage through bactericidal serum cultures. Teague and McWilliams state that they found some changeable bacilli, but they do not draw any definite conclusions from their finding.

According to our observation colonies of many strains of the colon-typhoid group may lose the property of spontaneous agglutination, while others that do not show this property at first may show it after some time.

From a stock culture we obtained colonies which were, and others which were not, agglutinated spontaneously. These were spread on plates of two per cent agar (using very high dilution of the bacteria in broth or salt solution so that each single colony should be separated from every other colony) and incubated for 24 hours at 37°C. The colonies thus grown were inoculated into broth tubes and placed in the incubator. Subcultures were made every two days by inoculating one loopful of the broth culture into a fresh tube of broth. The bacilli were examined after each sub-culture by streaking a loopful of the freshly inoculated broth on agar plates. The following results on agar plates and in cultures were obtained:

TABLE 2

Broth cultures of the isolated colony were kept at 37°C., and transplanted every second day, then examined for any change of colonies by streaking on agar plates

ORGANISM	NUMBER OF STRAINS	TYPE OF COLONY	NUMBER OF DAYS, WHEN CHANGE FOUND. REPEATED ONE TO THREE TIMES		
Bact. typhosum	No. 1	Non-spont. aggl.	24	35	
		Spont. aggl.	10	13	
	No. 2	Non-spont. aggl.	6	6	13
		Spont. aggl.	16	14	5
	No. 29	Non-spont. aggl.	32	12	20
		Spont. aggl.	50	48	47
Bact. paratyphosum A	No. 1	Non-spont. aggl.	4	4	3
		Spont. aggl.	4	4	4
	No. 2	Non-spont. aggl.	3	3	3
		Spont. aggl.	−30		
	No. 3	Non-spont. aggl.	7	6	8
		Spont. aggl.	12	18	15
Bact. paratyphosum B	No. 4	Non-spont. aggl.	5	5	4
		Spont. aggl.	13	25	
	No. 5	Non-spont. aggl.	5	10	
		Spont. aggl.	−60		
	No. 6	Non-spont. aggl.	5	8	12
		Spont. aggl.	24	25	
Bact. coli	No. 1	Non-spont. aggl.	2	3	3
		Spont. aggl.	2	3	2
	No. 2	Non-spont. aggl.	7	4	
		Spont. aggl.	7	10	
	No. 3	Non-spont. aggl.	10	13	
		Spont. aggl.	30	28	
Bact. enteritidis	No. 2	Non-spont. aggl.	24		
		Spont. aggl.	16		

TABLE 2—*Continued*

ORGANISM	NUMBER OF STRAINS	TYPE OF COLONY	NUMBER OF DAYS, WHEN CHANGE FOUND. REPEATED ONE TO THREE TIMES		
Bact. enteritidis	No. 3	Non-spont. aggl.	7		
		Spont. aggl.	5		
	No. 7	Non-spont. aggl.	5		
		Spont. aggl.	5		
Bact. dysenteriae	Shiga No. 1	Non-spont. aggl.	22		
		Spont. aggl.	4	5	
	Flexner No. 1	Non-spont. aggl.	8		
		Spont. aggl.	17		

Key, – No change.

(*a*) Different organisms showed a differentiation into two types of colony at different periods of growth, *Bact. coli* showing a change in from 2 to 30 days.

(*b*) *Bact. paratyphosum* A showed the change within 3 to 14 days (some strains however, remaining constant even after 30 days).

(*c*) *Bact. paratyphosum* B showed a transition period of from 4 to 30 days, while some strains did not show the spontaneously agglutinated type of colony even after 60 days.

(*d*) *Bact. dysenteriae* group showed changes in from 4 to 22 days (some strains being negative after 30 days).

(*e*) *Bact. enteritidis* changed in from 5 to 24 days.

(*f*) *Bact. typhosum* showed the longest period, the time varying from 6 to 50 days in some strains, while others did not show changes even after 3 months or more.

From colonies of known type (as determined above) isolated on agar plates, the different members of the colon-typhoid group were inoculated into broth media and allowed to grow at room temperature without sub-culture, plates being made every five or ten days to determine the type of colony. In 33 strains of *Bact. typhosum* of both types, changes were observed to take place in 30 strains in fifteen to ninety days, while three strains,

originally of a spontaneously agglutinating variety, showed no change after one hundred days. Eighteen strains of *Bact. paratyphosum* A showed changes in from ten to eighty-five days, two strains of an agglutinating type, showed no change until after one hundred days. Nineteen strains of *Bact. paratyphosum* B. showed changes in from fifteen to one hundred days, while two strains of spontaneously agglutinating type did not change even in one hundred days. All strains of *Bact. coli* showed a change in from ten to thirty days. *Bact. enteritidis* was examined after 60 days growth, and all 8 strains showed a change. With the *Bact. dysenteriae* group two strains showed changes in thirty-five to forty days, while two strains showed no change after sixty days.

The period at which the change in colony occurs depends upon the strains; some strains always change within a short time and others only after many days.

ISOLATION OF TYPES OF COLONY FROM STOCK CULTURES (TABLE 3)

In our first work with stock cultures three types were found. These were, pure colonies not showing spontaneous agglutination, pure colonies showing spontaneous agglutination and mixed colonies showing the presence of both types.

In a study of the colon-typhoid group of bacilli from agar stab cultures which had ordinarily been sub-cultured at intervals of two or three months, there were found many cultures producing colonies of only one kind, namely those which agglutinated spontaneously; other cultures yielded colonies of bacilli all of which failed to agglutinate spontaneously.

In the present work extending over a period of a year, it was found that each pure type after frequent transplantation tended to change into both types; of 29 strains of *Bact. typhosum* showing pure colonies of the spontaneous agglutinating type only 3 strains remained unchanged. Of 4 originally pure strains of *Bact. coli* used only 2 adhered to the original type. *Bact. paratyphosum* A (19 strains) *Bact. enteritidis* (9 strains) and *Bact. dysenteriae* (3 strains) showed both types of colonies. *Bact. paratyphosum* B (21 strains) showed both types of colonies, but

2 strains (nos. 19 and 21), after frequent sub-culture in broth, pepton water and agar throughout a period of one year, gave 1 type of spontaneously agglutinating bacilli during the entire period. When, however, 1 per cent glucose broth was used for daily transplants, culture no. 19 showed 2 types of bacilli after seven days and culture no. 21 after eighteen days.

A new strain of *Bact. typhosum* was isolated from the blood of a patient at the Peter Bent Brigham Hospital in the spring of

TABLE 3

Isolation of colonies from stock stab cultures of agar medium

	ORGANISM	NUMBER OF STRAINS	SPONTANEOUS AGGLUTINATION	NON-SPONTANEOUS AGGLUTINATION	MIXED, SHOWING BOTH TYPES	DOUBTFUL AGGLUTINATION
First isolation	*Bact. typhosum*.....	33	4	15	13	1
	Bact. paratyphosum A.....	19	5	7	7	
	Bact. paratyphosum B.....	23	4	3	16	
	Bact. coli.....	8	1	3	4	
	Bact. enteritidis....	9	1	2	6	
	Bact. dysenteriae...	3	—	1	2	
Isolation of subcultures after several months	*Bact. typhosum*.....	33	3	—	29	1
	Bact. paratyphosum A.....	19	—	—	19	
	Bact. paratyphosum B.....	25	—	—	23	
	Bact. coli.....	8	—	2	6	
	Bact. enteritidis	9	—	—	9	
	Bact. dysenteriae...	3	—	—	3	

1918. The cultures on agar plates gave pure smooth colonies which when transplanted to broth showed non-spontaneously agglutinated bacilli. A single colony was fished from an agar plate and transplanted successively for a period of twelve days in broth. At the end of the twelfth day it showed the presence of bacilli of the spontaneously and non-spontaneously agglutinating types.

Most of the colonies which developed after planting cultures freshly obtained from animals showed non-spontaneous-agglu-

tination. When these were sub-cultured at later periods, the colonies showed both agglutinating and non-agglutinating types.

In all instances the changes in the strain was from a pure to two types of colonies. The results of this work indicate that every member of the colon-typhoid group is changeable and may develop two types of colonies, dependent upon duration of cultivation or other circumstances of growth, as well as upon the type of artificial culture medium employed.

PHENOMENA OF SPONTANEOUS AGGLUTINATION IN VARIOUS FLUID CULTURE MEDIA (TABLE 4)

It has been observed, that spontaneous agglutination may occur in all ordinary fluid or solid culture media. Spontaneous agglutination occurs in a greater degree in glycerol broth, pepton water and all acid media, than it does in neutral or alkaline broth and it is more marked in concentrated than in dilute, liquid pepton medium. Cultures in alkaline broth usually remain clear and transparent, the presence of the alkali greatly retarding or completely inhibiting spontaneous agglutination.

When glucose broth was used as a culture medium, spontaneous agglutination of *Bact. typhosum* was prevented in many instances, but glucose broth cultures of *Bact. paratyphosum* A and B showed spontaneous agglutination in 24 hour cultures, and this has been even more marked with similar cultures of *Bact. coli.*

Growth in plain broth, either weakly acid or neutral in reaction, depends upon the type of colony. In some instances there is an excessive growth as well as a spontaneous agglutination. When such a culture was shaken or mixed with broth or salt solution, it disintegrated being thus differentiated from true spontaneous agglutination, in which disintegration occurs only when special methods are employed.

Plain broth or pepton water cultures which became spontaneously agglutinated were at first clear and after twenty-four hours a sediment was forming. If a cloudy growth is encountered in sub-culture, we are sure to find both spontaneous and non-spontaneous agglutination types of organisms present. This

TABLE 4

Spontaneous agglutination on various culture media

SPONTANEOUS AGGLUTINATION OF CONTROL IN PLAIN BROTH	BACT. TYPHOSUM									BACT. PARATYPHOSUM A									BACT. PARATYPHOSUM B									BACT. COLI								
	−				+				Total	−				+				Total	−				+				Total	−				+				Total
Number of strains	No. 1	No. 2	No. 4	No. 5	No. 1, 2	No. 2, 2	No. 4, 2	No. 7, 2	8	No. 1	No. 2	No. 3	No. 4	No. 1, 2	No. 2, 2	No. 3, 2	No. 4, 2	8	No. 1	No. 2	No. 3	No. 4	No. 1, 2	No. 2, 2	No. 3, 2	No. 4, 2	8	No. 1	No. 2	No. 3	No. 6	No. 1, 2	No. 2, 2	No. 3, 2	No. 4, 2	8
Plain broth	−	−	−	−	1	3	2	1	7	−	−	−	−	2	2	1	2	7	−	−	−	−	3	2	2	2	9	−	−	−	−	2	2	2	2	8
One per cent glycerol broth	−	2	−	−	2	3	2	1	10	−	−	−	−	2	2	2	3	9	1	−	−	−	3	2	3	3	12	−	−	−	−	2	2	2	2	8
One per cent glucose broth	−	−	−	−	1	2	−	−	3	2	3	3	−	2	2	2	2	16	2	2	1	2	2	2	2	3	16	3	2	3	3	3	3	3	3	23
One per cent pepton water	−	2	−	−	2	3	2	1	10	1	−	−	−	2	3	2	3	11	1	1	−	1	3	2	3	3	14	1	1	1	−	2	2	2	2	11

Key: 3 strong; 2 medium; 1 weak agglutination; − Negative; + Spont. agglutinated.

can be determined by plating the culture and observing the two types of colonies. If flocculi, pellicles or small clumps are present in a twenty-four hour sub-culture of a non-spontaneously-agglutinating strain, we can assume that it has changed its characteristics and expect to find both types of growth present.

VARIATION IN GROWING POWER OF THE SPONTANEOUS AND NON-SPONTANEOUS AGGLUTINATION COLONIES FROM ONE STRAIN

Dreyer, using stock cultures for the agglutination test, passed a strain through several sub-cultures on broth medium and then treated a vigorously growing sub-culture with formalin. He reports that agglutination reactions with such cultures are more powerful.

Block (1897) claims that if too frequent transplantation of the culture is made there will occur in time spontaneous agglutination. This is possible only if an easily changeable strain containing spontaneous and non-spontaneous agglutination types is used. After isolating the non-agglutinating type of colony two broth cultures were made from a single colony, one of which was daily transplanted into fresh broth. After several days in the agar plate from the sub-cultures many colonies of spontaneously agglutinating types were present, while in the first broth culture which was not transplanted, there were only a few colonies of the spontaneously agglutinating types. Our deduction from these experiments is, that the type of organism showing spontaneous agglutination grows more vigorously than the non-spontaneously-agglutinating type taken from the strain in these experiments. On the other hand, if the growth of the non-spontaneously-agglutinating strain is stimulated, one will find that the spontaneously agglutinating type is weakened greatly and, in some cases, entirely disappears.

Experiments were made on growing the two types of culture together in broth, as follows: Broth cultures were prepared containing one loopful of a spontaneous and one, of a non-spontaneous agglutination type of organism. The results as shown on agar plates made at the end of each twenty-four hours varied widely. In some the growth of non-spontaneously agglutinating

bacilli daily exceeded that of spontaneously agglutinating bacilli; others showed a more vigorous growth of the latter type, and a few remained constant, showing parallel growth of the two types.

Other cultures which were not transplanted, showed a very slow change, while the daily transplanted culture showed in comparison very rapid changes in the proportion of the colony types. This varied directly with the original strains of the bacilli.

SPECIFIC AGGLUTINATING POWER BETWEEN TWO TYPES OF COLONIES OF ONE STRAIN

In experiments on specific agglutination with different types of colonies, no difference was found in the power of agglutination with two types of colonies of one strain.

Spontaneous agglutination differs chemically from specific serum agglutination, in that spontaneous agglutination can be prevented with most strains of *Bact. typhosum* and *Bact. paratyphosum* A and to a lesser extent with *Bact. paratyphosum* B, by adding 0.05 to 1 per cent formalin. Formalin in 0.05 to 0.2 per cent, does not interfere with specific serum agglutination, but slightly increases it if plain salt solution (0.85 per cent) has been used.

Many spontaneously agglutinating strains of *Bact. typhosum* when grown in glucose broth fail to show spontaneous agglutination. In others spontaneous agglutination is not inhibited by this means; neither does growth in glucose broth destroy the power of specific agglutination.

NOTES ON AGGLUTINATION TEST

In the agglutination test as applied to members of the colon-typhoid group it is necessary to differentiate between organisms giving spontaneous agglutination and organisms which do not agglutinate spontaneously. Organisms of the first group appear to give specific serum agglutination, but in the control bacillary suspension, when mixed with broth, pepton water,

salt solution or tap water (the diluents usually employed), they also give spontaneous agglutination.

Even old stock cultures which fail to give spontaneous agglutination at first, will do so, if the culture is inoculated into fresh media or repeatedly sub-cultured on agar, pepton water or broth. Spontaneous agglutination also appears in the suspension of a twenty-four-hour agar culture in water, salt solution, broth, pepton water or diluted serum.

If we examine an easily changeable stock culture, i. e.,a culture containing both types of colonies, and streak such a culture on the agar plate, we will find the two types of colonies described above.

If upon plating we can isolate colonies of the non-spontaneously agglutinating type, only occasional sub-cultures from these colonies have to be made for the agglutination test, as such colonies invariably fail to agglutinate spontaneously, and further tests are unnecessary.

Agar cultures seem to show greater stability than fluid media cultures; some strains, however, always show one type of colony. For example certain strains of *Bact. typhosum* which have been handled for a year and a half showed constantly on both solid and fluid media non-spontaneous agglutination. Such a strain is valuable as a stock culture for the Widal test. In many of these cultures, even after being kept for several months in an ice chest, there was only one type of colony present corresponding with the original form, although some of the cultures were dead. These cultures were made on 0.5 per cent agar medium and the tubes were sealed with paraffin.

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THE SOURCES AND CHARACTERISTICS OF THE BACTERIA IN DECOMPOSING SALMON

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In the course of an extensive investigation of the bacteriology of decomposing salmon a large number of cultures from various sources have been collected for study. The descriptions and, to some extent, the identification of the bacteria obtained from decomposing salmon caught on the spawning migration have been given in a previous report on this subject (Hunter, 1920). In that report the statement was made that the bacteria isolated from decomposing salmon were found to be those which are described in the literature as water, sewage and soil organisms. It was also stated that there was apparently no contamination of the fish with spore-forming organisms in the cannery. In order to determine definitely the relation between the flora of sea-water and that of decomposing salmon, experiments on the decomposition of "feedy" salmon, which have been described in a previous report (Hunter, 1921) were conducted at Astoria, Oregon. From the plates and the mixed cultures obtained from the decomposing salmon 197 cultures were selected for study.

Samples of sea-water were collected from various locations near the mouth of the Columbia River. From these water samples, plated on glucose agar, 14 cultures were selected for study. Although this number of cultures appears rather small,

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it may be taken as representative of the flora of the sea-water in that locality. Particular care was taken to avoid the selection of any more duplicates than was necessary and, since the plates from the water samples presented the same types of colonies repeatedly, the 14 cultures collected seemed fairly representative.

In order to extend this investigation of the bacterial flora of the salmon industry, a field laboratory was later established at Juneau, Alaska. Samples of sea-water were collected from various locations in southeastern Alaska. A large number of mixed cultures from different parts of the salmon canneries in this region were also collected to determine whether or not the bacterial flora of the cannery is identical with that of the sea-water and that of the decomposing salmon. From the water samples collected in Alaska 11 cultures were selected for further study. Here again the different types of colonies on the plates from the water samples were comparatively few and the 11 cultures obtained seemed representative of the bacterial flora of the sea-water in that region. From the mixed cultures obtained from the canneries 94 pure cultures were isolated and preserved for further study.

The organisms from sea-water and decomposing salmon and from the Alaskan canneries have been studied as separate groups and according to their morphology and their cultural reactions duplicates have been checked within each group. This has reduced the original number of 316 cultures to 85. The final designation of each of the 85 cultures with the number of original cultures included under this designation are given in table 1.

Each of the 85 cultures has been studied regardless of its action on salmon but particular attention has been given to the character which each organism may or may not possess of producing foul odors or indol in a specially prepared fish medium.[2]

[2] This medium was prepared in the following manner:

To 1000 grams of finely chopped saltwater trout, or weakfish, from which the skin and bones had been removed, was added 1000 cc. of distilled water and 15 grams of pepton. The infusion was made by heating in the Arnold sterilizer or on a water bath at 95° to 100°C. for one hour with occasional stirrings. The juice was strained through cheese cloth with a meat press, filtered through cotton and the reaction adjusted to neutral. The infusion was then heated in the

TABLE 1

Summary of the number of cultures studied

CULTURE	NUMBER OF ORIGINAL CULTURES INCLUDED UNDER THIS DESIGNATION	CULTURE	NUMBER OF ORIGINAL CULTURES INCLUDED UNDER THIS DESIGNATION	CULTURE	NUMBER OF ORIGINAL CULTURES INCLUDED UNDER THIS DESIGNATION
W_1	2	360	11	443	2
W_{1a}	1	366	1	444	3
W_2	4	374	12	451	1
W_3	3	380	12	452	2
W_{3a}	1	390	7	452_a	1
W_{3b}	1	395	1	453_a	1
W_6	1	397	1	454	3
		399	1	456	9
W_{6a}	1	400	1	458	2
W_{7a}	2	401	1	459_a	1
W_{7b}	1	403	1	460	7
W_{7c}	1	406_a	2	461	1
W_{8b}	1	410	3	462	2
W_{12}	2	416	4	463	3
		417	1	463_a	1
W_{12a}	1	419	1	464	3
W_{13}	3	420_c	1	466	2
		420E	1	467	1
H_2	1	421	1	470_a	1
I	1	424	3	471_a	1
301_a	3	425	4		
303	4	427	2	473	2
314_a	3	429	1	480	1
316b	1	429_a	1	503	6
323	5	430	8	510	7
325	52	431_a	1	513_a	1
343	3	432	1	516	30
351_a	1	436_a	2	518	17
354	1	439	4	523	5
		440	5	525	5

Each organism was grown in this medium for one week at 30°C. and the presence or absence of abnormal odors noted. The morphology and cultural reactions of 79 of the 85 cultures are

Arnold for thirty minutes at 100°C. and filtered, using folded filter papers. About 10 cc. was placed in each tube with about 1.5 grams of raw fish. For anaerobic cultures the surface was covered with a layer of liquid petrolatum. The medium was sterilized in the autoclave for fifteen minutes at 15 pounds.

given in tables 2, 3, 4, 5, 6, 7, 8 and 9. For convenience in presentation, the cultures from sea-water, salmon and the Alaskan canneries are given separately. The cultures from sea-water are given in tables 2 and 3 according to their ability to liquefy gelatin and regardless of pigment production. The cultures from decomposing salmon are described in tables 4, 5 and 6. Table 4 contains the gelatin-liquefying bacteria exclusive of pigment producers, table 5 the gelatin non-liquefying bacteria exclusive of pigment producers and table 6 the pigment producing bacteria. The cultures from the Alaskan canneries are described in tables 7, 8 and 9, and are divided according to gelatin liquefaction and pigment production in the same manner as those presented in tables 4, 5 and 6.

In recording the odor produced by the individual bacteria in fish broth three terms are used. The term "normal" signifies that no abnormal odor was produced and that the organism alone has no physically discernible effect on the fish. The term "off" is used to describe any abnormal odor which is not putrid or exactly foul but indicates that decomposition is taking place. The use of the term "foul" is obvious. While some of the organisms alone had no apparent effect on the fish, it was possible that these organisms, when grown in fish broth in mixed culture with other organisms might aid in the decomposition. With this in mind several combinations of bacteria were inoculated into fish broth in flasks and incubated for one week at 30°C. All the cultures from sea-water were inoculated into one flask and in one week produced a distinctly putrid odor. Four different combinations of bacteria, the sources of which were decomposing salmon, produced an extremely putrid, foul odor in the fish broth. A mixed culture of all the organisms isolated from the canneries produced a foul, but not a distinctly putrid, odor in this medium. The odor produced by the organisms growing in combination was in each case much worse than when grown individually.

Six cultures which have been carried along as individual cultures are not included in tables 2 to 9. These are 351_a, a pink yeast; 451, 459_a, 470_a and 480, white yeasts; and 366, a culture

of Actinomyces. Culture 351_a was isolated from the flesh of the back of a decomposing salmon and 366 was obtained from the intestines of a salmon. Cultures 451, 459_a and 480 were isolated from canneries in Ketchikan, Alaska, and 470_a was obtained from the cannery at Yes Bay, Alaska. Of the 79 cultures described in tables 2 to 9, 72 are rod-shaped organisms without spores, 3 are spore-forming rods and 4 are streptococci.

In studying these bacteria but little attempt has been made to identify as specific organisms any except the lactose fermenting organisms and the fluorescent bacteria. The attempt has been rather to determine what relation, if any, exists between the sea-water flora and the flora from decomposing salmon and from the canneries. The extensive work done by several groups of investigators, notably Winslow, Kligler and Rothberg (1919) and Levine (1918), make it possible to identify members of the colon-aerogenes group and, as far as possible, the lactose fermenting bacteria in this collection have been identified. The descriptions of fluorescent bacteria given by Edson and Carpenter (1912) and by Tanner (1918) also make it possible to identify bacteria of this group. The inadequate descriptions of non-fermenting, asporogenous, gelatin liquefying and non-liquefying bacteria given in the literature, if they are given at all, make it very difficult to identify organisms of this kind.

Examination of table 2 shows that of the 9 gelatin liquefying cultures from sea-water 1 is *Bact. cloacae* and 2 are *Ps. fluorescens*. W_3 differs from W_2 only in its failure to reduce nitrates. Two cultures in this table are unpigmented forms fermenting none of the sugars; one of the cultures, W_{13}, produces spores. Two cultures are pigmented organisms which show no fermentative reactions. One culture, W_{13}, produces spores and ferments glucose and sucrose with the production of acid but no gas and one culture, W_{7c}, ferments glucose, lactose and sucrose with the production of acid.

In table 3 one culture, W_{12}, was identified as *Bact. aerogenes*. One culture produces an acid fermentation in glucose; two cultures are unpigmented bacteria which do not ferment and two cultures produce yellow pigment and show no fermentative reactions.

TABLE 2

Gelatin liquefying bacteria from sea-water

CULTURE	SOURCE	SHAPE	GRAM STAIN	LITMUS MILK	BIOCHEMICAL REACTIONS							FISH BROTH	PIGMENT	TYPE RESEMBLED
					Indol	Nitrate reduced	Glucose	Lactose	Sucrose	Methyl red	Voges-Proskauer			
W_{1a}	Water from Ilwaco, Wash.	Small rods	0	Peptonized	+	+	0	0	0	0	0	Off	White	
W_{2a}	Water from Ilwaco, Wash.	Medium size rods	0	Peptonized	0	+	0	0	0	0	0	Foul	Flesh color	
W_{1s}	Water from Ilwaco, Wash.; Haines, Alaska; Lynn Canal, Alaska	Large rods with spores	0	Peptonized	0	+	0	0	0	0	0	Normal	White	
W_{2b}	Water from Lynn Canal, Alaska	Small rods	+	Decolorized	0	+	0	0	0	0	0	Off	Orange	
W_2	Water from Ilwaco, Wash., 4 miles off Oregon Coast; Lynn Canal, Alaska	Medium size rods	0	Peptonized	+	+	A*	0	0	0	0	Off	Fluorescent	*Ps. fluorescens*

W_s	Water from Ilwaco, Wash.; 4 miles off Oregon Coast; Tee Harbor, Alaska	Medium size rods	0	Peptonized	0	+	A	0	0	0	0	Off	Fluorescent	*Ps. fluorescens*
W_1	Water from Ilwaco, Wash., and 4 miles off Oregon Coast	Large rods and spores	0	Peptonized	0	+	A	0	A	0	0	Normal	White	
W_{2c}	Water from Haines, Alaska	Medium size rods	0	No change	+	+	A	A	A	0	0	Normal	White	
W_4	Water from Chinook, Wash.	Medium size rods	0	Coagulated, peptonized	+	+	AG†	AG	AG	0	+	Foul	White	*Bact. cloacae*

* A indicates acid but no gas.

† AG indicates acid and gas.

TABLE 3

Gelatin non-liquefying bacteria from sea-water

CULTURE	SOURCE	SHAPE	GRAM STAIN	LITMUS MILK	BIOCHEMICAL REACTIONS							FISH BROTH	PIGMENT	TYPE RESEMBLED
					Indol	Nitrate reduced	Glucose	Lactose	Sucrose	Methyl red	Voges-Proskauer			
W_{2B}	Water from Ilwaco, Wash.	Medium size rods	+	Decolorized	0	+	0	0	0	0	0	Normal	Yellow	
W_{6a}	Water from Chinook, Wash.	Small rods	+	No change	0	+	0	0	0	0	0	Normal	Yellow	
W_{7B}	Water from Haines, Alaska	Small rods	0	Decolorized	0	+	0	0	0	0	0	Off	White	
W_{12a}	Water from Funter Bay, Alaska	Short, thick rods	0	No change	+	+	0	0	0	0	0	Normal	White	
W_{7a}	Water from Haines, Alaska, and Tee Harbor, Alaska	Small rods	0	No change	0	+	A*	0	0	0	0	Normal	White	
W_{12}	Water from Tee Harbor, Alaska, and Funter Bay, Alaska	Medium size rods	0	Acid coagulated	0	+	AG†	AG	AG	0	+	Off	White	*Bact. aerogenes*

* A indicates acid but no gas.

† AG indicates acid and gas.

Of the 10 cultures described in table 4, 3 were identified as *Bact. cloacae* although in the case of 323 and 380 the methyl-red and the Voges-Proskauer tests are not typical. Repeated plating and testing of these two cultures did not alter the results of these tes 1 323 and 380 have necessarily been recorded as atypical strains. Culture 316B cannot be confirmed as *Bact. cloacae* since it does not ferment sucrose. In all other reactions it is similar to *Bact. cloacae*. One culture, 397, in table 4 is a spore-forming organism which does not ferment any of the sugars; one culture, 395, ferments glucose with the production of acid but no gas; one culture, 354, produces acid in glucose and sucrose and three cultures, 399, 400 and 513_a, produce acid in glucose, lactose and sucrose. Culture 513_a is a streptococcus.

In table 5 one culture, 525, was identified as *Bact. communior*, one, H_2, as *Bact. aerogenes* and two, 343 and 523, as *Bact. coli*, although 343 is atypical in that it gives a positive result in both the methyl-red and the Voges-Proskauer reactions. Culture 503 produces acid and gas in glucose and gives a positive methyl-red test. Three cultures are streptococci, two of which, 516 and 518, produce acid in glucose, lactose and sucrose and one of which, 510, produces acid only in glucose and lactose. One culture, 406, produces an acid fermentation in glucose. The two remaining cultures in this table, 314_a and 410, show no fermentative reactions.

Among the pigment producing bacteria described in table 6 are two strains of *Ps. fluorescens*, 325 and 374. Culture 374 differs from 325 only in its failure to produce indol. Four of the cultures in this table show no fermentative reactions. Culture 401 produces acid in glucose and culture 403 has an acid fermentation in glucose, lactose and sucrose.

Of the 11 cultures from canneries described in table 7, two, 417 and 419, were identified as *Bact. cloacae*, although 419 must be recorded as atypical inasmuch as it gives negative results with both the methyl-red and the Voges Proskauer reactions. Two cultures, 471_a and 473, produce acid in glucose, lactose and sucrose and one culture, 429, produces an acid fermentation in glucose only. The remaining six cultures in this table show no fermentative reactions.

TABLE 4

Gelatin liquefying bacteria from salmon exclusive of pigment producers

CULTURE	SOURCE	SHAPE	GRAM STAIN	LITMUS MILK	BIOCHEMICAL REACTIONS							FISH BROTH	TYPE RESEMBLED
					Indol	Nitrate reduced	Glucose	Lactose	Sucrose	Methyl red	Voges-Proskauer		
397	Stomach	Large rods with spores	0	Peptonized	0	+	0	0	0	0	0	Normal	
395	Heart	Large rods	0	Coagulated	0	+	A*	0	0	0	0	Off	
354	Intestines	Small rods	0	Peptonized	+	+	A	0	A	0	0	Off	
399	Blood	Medium size rods	0	Decolorized	+	+	A	A	A	0	0	Off	
400	Mouth	Small rods	0	Acid-coagulated	0	0	A	A	A	0	0	Off	
513_a	Back	Streptococci	+	No change	0	+	A	A	A	+	0	Normal	
316_b	Belly	Small rods	0	Coagulated, peptonized	+	+	AG†	AG	0	0	+	Very foul	
323	Pew wound, belly, stomach	Medium size rods	0	Acid-coagulated, peptonized	+	+	AG	AG	AG	+	+	Foul	*Bact. cloacae*
380	Back, belly, pew wound, mouth, intestines, heart, liver	Short, thick rods	0	Acid-coagulated, peptonized	0	+	AG	AG	AG	+	0	Off	*Bact. cloacae*
390	Belly, pew wound, intestines, kidney	Medium size rods	0	Acid-coagulated, peptonized	0	+	AG	AG	AG	0	+	Foul	*Bact. cloacae*

* A indicates acid but no gas.

† AG indicates acid and gas.

TABLE 5

Gelatin non-liquefying bacteria from salmon exclusive of pigment producers

CULTURE	SOURCE	SHAPE	GRAM STAIN	LITMUS MILK	BIOCHEMICAL REACTIONS							FISH BROTH	TYPE RESEMBLED
					Indol	Nitrate reduced	Glucose	Lactose	Sucrose	Methyl red	Voges-Proskauer		
314_a	Back, belly, intestines	Small rods	0	No change	0	+	0	0	0	0	0	Normal	
410	Pew wound, blood, gills	Short thick rods	0	No change	0	0	0	0	0	0	0	Normal	
406	Mouth, belly, intestines	Short thick rods	0	No change	0	+	A*	0	0	0	0	Normal	
510	Back, belly, stomach	Streptococci	+	Decolorized	0	0	A	A	0	0	0	Off	
516	Back, belly, pew wound, intestines, stomach, kidney	Streptococci	+	Acid	+	0	A	A	A	+	0	Normal	
518	Back, belly, pew wound, intestines	Streptococci	+	Acid	0	0	A	A	A	+	0	Normal	
503	Back, belly	Medium size rods	0	Acid	0	+	AG†	0	0	+	0	Foul	
343	Pew wound, back, stomach	Small rods	0	Acid	+	+	AG	AG	0	+	+	Off	*Bact. coli*
523	Back, pew wound, stomach	Medium size rods	0	Acid	+	+	AG	AG	0	+	0	Foul	*Bact. coli*
H_2	Pew wound	Medium size rods	0	Acid	0	+	AG	AG	AG	0	+	Normal	*Bact. aerogenes*
525	Belly, pew wound, intestines	Medium size rods	0	Acid	+	+	AG	AG	AG	+	0	Normal	*Bact. communior*

* A indicates acid but no gas.

† AG indicates acid and gas.

One culture, 424, in table 8 was identified as *Bact. aerogenes;* one culture, 464, produces acid in glucose and sucrose and two cultures, 454 and 463, produce acid in glucose only. The other eight cultures described in this table do not ferment any of the sugars.

Among the twelve cultures in table 9 are two, 431_a and 467, which produce acid in glucose, lactose and sucrose; one, 461, which produces acid in glucose and lactose and one, 443, which produces acid in glucose only. The remaining 8 cultures show no fermentative reactions.

Throughout the investigation no obligate anaerobes were isolated. Anaerobic cultures were made from the material investigated and several cultures isolated which were at first regarded as anaerobic bacteria but subsequent work showed them to be facultative. This was especially true of the streptococci isolated from salmon. These streptococci were originally isolated from anaerobic cultures and they appear to grow best under anaerobic conditions. They do grow fairly well on agar slants, however, and have been carried along in this work as aerobic organisms.

As a result of this study of the morphology and the cultural reactions of these 79 organisms it has been possible in several instances to identify as the same organism cultures from the various sources and in this way to establish a partial correlation between the bacterial floras of sea-water, of decomposing salmon and of the Alaskan canneries. This has been done where the morphology and cultural reactions are such that there can be no doubt that the cultures are identical even though it were impossible to identify them as to group or species. This correlation is shown in table 10.

Cultures W_2 and 325 are both the same strain of *Ps. fluorescens.* Reference to table 1 will show the relative abundance of this organism among the original 316 cultures. This organism appears to be widely distributed in the sea-water since it was obtained from samples collected at Ilwaco, Wash., four miles off the Oregon Coast and Lynn Canal, Alaska (see map, fig. 1). It was also obtained 52 times from the various parts of

TABLE 6
Pigment producing bacteria from salmon

TABLE 6

Pigment producing bacteria from salmon

CULTURE	SOURCE	SHAPE	PIGMENT	GRAM STAIN	GELATIN LIQUEFIED	LITMUS MILK	BIOCHEMICAL REACTIONS							FISH BROTH	TYPE RESEMBLED
							Indol	Nitrate reduced	Glucose	Lactose	Sucrose	Methyl red	Voges-Proskauer		
I	Pew wound	Small rods	Orange	0	0	Decolorized	0	+	0	0	0	0	0	Normal	
301a	Back, pew wound	Small rods	Yellow	+	0	No change	0	+	0	0	0	0	0	Normal	
303	Back, belly	Small rods	Lemon yellow	+	0	Decolorized	0	+	0	0	0	0	0	Normal	
360	Back, belly, pew wound, stomach	Small rods	Flesh color	0	+	Peptonized	+	+	0	0	0	0	0	Foul	
325	Back, belly, pew wound, stomach, intestines, gills, mouth, liver, kidney	Medium size rods	Fluorescent	0	+	Peptonized	+	+	A*	0	0	0	0	Off	*Ps. fluorescens*
374	Back, pew wound, stomach, intestines, heart, liver, blood	Medium size rods	Fluorescent	0	+	Peptonized	0	+	A	0	0	0	0	Off	*Ps. fluorescens*
401	Gills	Medium size rods	Orange	0	+	Peptonized	0	+	A	0	0	0	0	Foul	
403	Liver	Small rods	Yellow	0	0	Decolorized	+	+	A	A	A	0	0	Off	

* A indicates acid but no gas.

TABLE 7

Gelatin liquefying bacteria from Alaska salmon canneries exclusive of pigment producers

CULTURE	SOURCE	SHAPE	GRAM STAIN	LITMUS MILK	BIOCHEMICAL REACTIONS							FISH BROTH	TYPE RESEMBLED
					Indol	Nitrate reduced	Glucose	Lactose	Sucrose	Methyl red	Voges-Proskauer		
416	Douglas Ketchikan	Medium size rods	0	Decolorized	0	+	0	0	0	0	0	Normal	
420_c	Haines	Small rods	0	Acid	0	+	0	0	0	0	0	Foul	
427	Ketchikan Excursion Inlet	Short, thick rods	0	Acid	0	0	0	0	0	0	0	Normal	
429_a	Excursion Inlet	Medium size rods	0	Decolorized	+	+	0	0	0	0	0	Foul	
432	Hoonah	Slender rods	+	Peptonized	0	+	0	0	0	0	0	Normal	
460	Sitka, Hawk Inlet, Ketchikan, Hoonah, Metlakatla	Small rods	+	Acid	0	+	0	0	0	0	0	Off	
429	Excursion Inlet	Short, thick rods	+	No change	+	+	A*	0	0	0	0	Normal	
471_a	Yes Bay	Small rods	0	Acid-coagulated	0	0	A	A	A	0	0	Off	
473	Yes Bay, Ketchikan	Medium size rods	0	Coagulated, peptonized	0	+	A	A	A	0	0	Normal	
417	Haines	Medium size rods	0	Coagulated, peptonized	0	+	AG†	AG	AG	0	+	Foul	*Bact. cloacae*
419	Haines	Medium size rods	0	Acid, peptonized	+	+	AG	AG	AG	0	0	Foul	*Bact. cloacae*

* A indicates acid but no gas.

† AG indicates acid and gas.

TABLE 8

TABLE 8

Gelatin non-liquefying bacteria from Alaskan salmon canneries exclusive of pigment producers

CULTURE	SOURCE	SHAPE	GRAM STAIN	LITMUS MILK	BIOCHEMICAL REACTIONS							FISH BROTH	TYPE RESEMBLED
					Indol.	Nitrate reduced	Glucose	Lactose	Sucrose	Methyl red	Voges Proskauer		
430	Excursion Inlet, Hoonah, Sitka, Ketchikan, Metlakatla	Small rods	0	Decolorized	0	+	0	0	0	0	0	Normal	
436_a	Hawk Inlet, Chomley	Medium size rods	0	Decolorized	0	+	0	0	0	0	0	Off	
439	Sitka	Small rods	0	Acid	0	+	0	0	0	0	0	Normal	
440	Sitka, Chomley, Metlakatla, Ketchikan	Medium size rods	0	Decolorized	0	0	0	0	0	0	0	Off	
452	Sitka, Ketchikan	Small rods	+	Acid	0	0	0	0	0	0	0	Normal	
452_a	Sitka	Medium size rods	0	No change	+	0	0	0	0	0	0	Off	
453_a	Ketchikan	Small rods	0	Decolorized	+	+	0	0	0	0	0	Off	
458	Ketchikan, Hawk Inlet	Small rods	0	Decolorized	+	+	0	0	0	0	0	Off	
454	Ketchikan	Short, thick rods	0	No change	0	+	A*	0	0	0	0	Normal	
463	Chomley, Yes Bay, Metlakatla	Short, thick rods	0	Decolorized	+	+	A	0	0	0	0	Normal	
464	Chomley, Yes Bay	Slender rods	0	Acid	0	0	A	0	A	+	0	Normal	
424	Haines, Tee Harbor	Medium size rods	0	Acid	0	+	AG†	AG	AG	0	+	Foul	*Bact. aerogenes*

* A indicates acid but no gas.

† AG indicates acid and gas.

TABLE 9

Pigment producing bacteria from Alaskan salmon canneries

CULTURE	SOURCE	SHAPE	PIGMENT	GRAM STAIN	GELATIN LIQUEFIED	LITMUS MILK	BIOCHEMICAL REACTIONS							FISH BROTH
							Indol	Nitrate Reduced	Glucose	Lactose	Sucrose	Methyl red	Voges Proskauer	
420_E	Haines	Small rods	Orange	0	0	Decolorized	0	+	0	0	0	0	0	Normal
421	Tee Harbor	Small rods	Flesh color	0	+	Peptonized	+	+	0	0	0	0	0	Foul
425	Hoonah, Chatham, Metlakatla, Excursion Inlet	Medium size rods	Orange	0	+	Decolorized	+	+	0	0	0	0	0	Normal
444	Hawk Inlet, Chatham, Sitka	Small rods	Lemon yellow	+	+	Slightly alkaline	0	+	0	0	0	0	0	Off
456	Hoonah, Chatham, Juneau, Excursion Inlet, Chomley, Ketchikan, Yes Bay	Medium size rods	Lemon yellow	+	0	No change	0	+	0	0	0	0	0	Foul
462	Chomley, Metlakatla	Small rods	Flesh color	0	+	Decolorized	+	+	0	0	0	0	0	Normal
463_a	Chomley	Short, thick rods	Yellow	0	0	Decolorized	0	+	0	0	0	0	0	Normal
466	Metlakatla, Ketchikan	Medium size rods	Lemon yellow	0	0	No change	0	0	0	0	0	0	0	Normal
443	Sitka, Chatham	Medium size rods	Orange	0	+	Peptonized	0	+	A*	0	0	0	0	Foul
461	Chomley	Medium size rods	Yellow	0	0	Decolorized	0	+	A	A	0	0	0	Normal
431_a	Hoonah	Short, thick rods	Lemon yellow	+	+	Coagulated	0	+	A	A	A	+	0	Normal
467	Metlakatla	Small rods	Yellow	0	0	Decolorized	+	+	A	A	A	0	0	Normal

* A indicates acid but no gas.

the salmon as described in table 10. Cultures W_3 and 374 are also identical strains of *Ps. fluorescens* isolated from sea-water in widely separated localities and from the various parts of decomposing salmon. As shown in table 1 this organism was obtained from water 3 times and from salmon 12 times. *Ps. fluorescens* was not found among the 94 cultures from Alaskan canneries.

Cultures W_{3a} and 301_a are yellow bacteria identical in their morphology and cultural reactions. This organism was obtained once from sea-water collected at Ilwaco, Wash., and three times from salmon. Cultures W_{6a}, 303 and 456 are also yellow pigment producing bacteria with identical morphology and cultural reactions isolated once from sea-water, four times from salmon and nine times from Alaskan canneries. References to table 10 and to the map (fig. 1) will show that this organism was found in widely separated areas throughout Southeastern Alaska. A third yellow organism, represented in the tables as 403 and 467, was obtained once from salmon and once from a cannery at Metlakatla.

Cultures W_6, 390 and 417 were identified as a strain of *Bact. cloacae* isolated once from water collected at Chinook, Wash., seven times from salmon and once from a cannery at Haines, Alaska (see map, fig. 1). Since 316_b, 323, 380 and 419 each represent an atypical strain of *Bact. cloacae* they have not been considered as identical with W_6, 390 and 417. It is possible, however, that the correlation might be extended to include the sources from which the atypical strains of this organism were isolated.

Cultures W_{7a}, 406 and 454 are identical and this organism was found twice in water from Alaska, twice from salmon and three times from canneries in Ketchikan, Alaska. It will be noted that Haines and Tee Harbor, Alaska, where this organism was found in the water are a great distance from Ketchikan (fig. 1) where it was found in the canneries. The fact that this organism was also isolated from salmon in Oregon indicates that it is widely distributed.

Cultures W_{7b} and 430 are bacteria having thes ame morphology and cultural reaction, isolated once from the water at Haines,

TABLE 10

Bacteria isolated from more than one source

CULTURE	WATER	SALMON	ALASKAN CANNERY	TYPE
W_2 325	Ilwaco, Wash. 4 miles off Oregon Coast, Lynn Canal, Alaska	Back, belly, pew wound, stomach, intestines, gills, mouth, liver, kidney		*Ps. fluorescens*
W_3 374	Ilwaco, Wash. Tee Harbor, Alaska, 4 miles off Oregon Coast	Back, pew wound, stomach, intestines, heart, liver, blood		*Ps. fluorescens*
W_{3a} 360 421	Ilwaco, Wash.	Back, belly, pew wound, stomach	Tee Harbor	
W_{3b} 301_a	Ilwaco, Wash.	Back, pew wound		
W_6 390 417	Chinook, Wash.	Belly, pew wound, intestines, kidney	Haines	*Bact. cloacae*
W_{6a} 303 456	Chinook, Wash.	Back, belly	Hoonah, Juneau, Excursion Inlet, Chatham, Chomley, Ketchikan, Yes Bay	
W_{7a} 406 454	Haines, Alaska Tee Harbor, Alaska	Mouth, belly	Ketchikan	

W_{7b} 430	Haines, Alaska		Excursion Inlet, Hoonah, Sitka, Ketchikan, Metlakatla	
W_{7c} 399 473	Haines, Alaska	Blood	Yes Bay, Ketchikan	
W_{12} H_3 424	Tee Harbor, Alaska Funter Bay, Alaska	Pew wound	Haines, Tee Harbor	*Bact. aerogenes*
W_{12a} 314_a 458	Funter Bay, Alaska	Back, belly, intestines	Ketchikan, Hawk Inlet	
W_{13} 397	Ilwaco, Wash. Haines, Alaska Lynn Canal, Alaska	Stomach		
$I–420_c$ $400–471_a$ 401–443 403–467		Pew wound Mouth Gills Liver	Haines Yes Bay Sitka, Chatham Metlakatla	

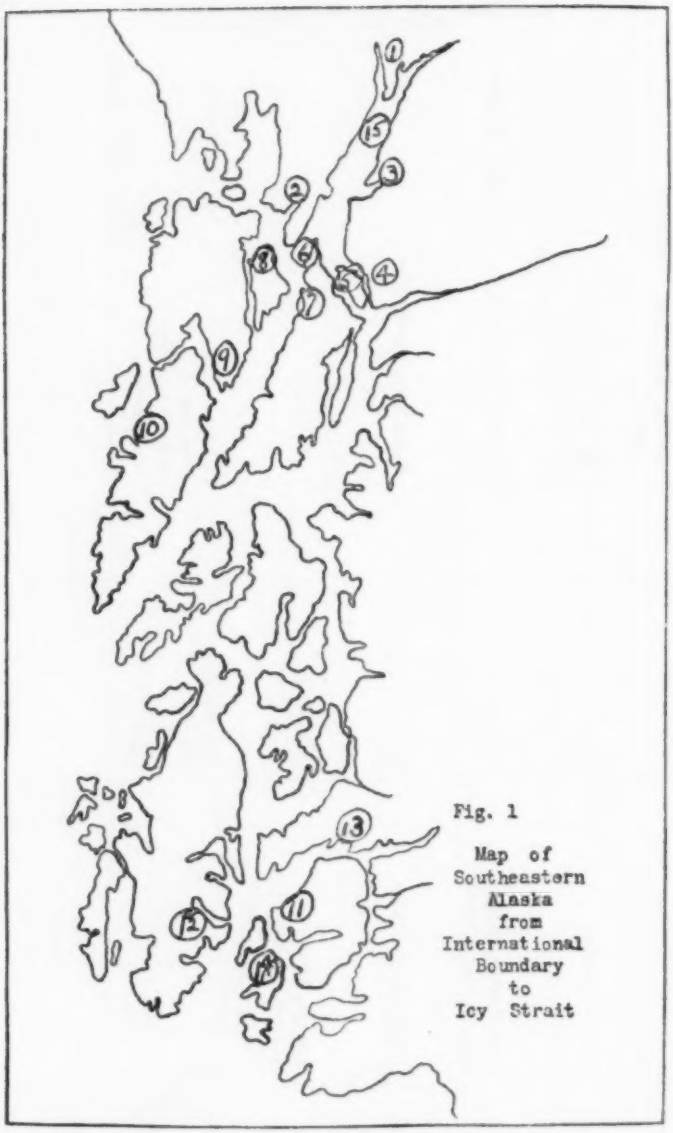

Fig. 1

Map of Southeastern Alaska from International Boundary to Icy Strait

1. Haines
2. Excursion Inlet
3. Tee Harbor
4. Juneau
5. Douglas
6. Funter Bay
7. Hawk Inlet
8. Hoonah
9. Chatham
10. Sitka
11. Ketchikan
12. Chomley
13. Yes Bay
14. Metlakatla
15. Lynn Canal

Alaska, and eight times from canneries in Alaska extending from Excursion Inlet on the north to Metlakatla on the south. This culture was not found in decomposing salmon.

As seen in the tables, cultures W_{7c}, 399 and 473 are identical in morphology and cultural reactions. This organism was isolated once from sea-water collected at Haines, Alaska, once from salmon and twice from canneries at Yes Bay and Ketchikan, Alaska.

Cultures W_{3a}, 360 and 421 were identified as a flesh-colored organism isolated once from sea-water collected at Ilwaco, Wash., eleven times from salmon and once from a cannery at Tee Harbor, Alaska.

The culture of *Bact. aerogenes* isolated twice from sea-water from Alaska, once from salmon and three times from canneries at Haines and Tee Harbor, Alaska, is designated in the tables under W_{12}, H_2 and 424.

Cultures W_{12a}, 314_a and 458 are identical. This organism was isolated once from sea-water from Funter Bay, Alaska, three times from salmon and twice from canneries at Ketchikan and Hawk Inlet. The isolation of this culture from material collected from such widely separated areas indicates that it is generally distributed throughout the whole region.

Only two spore-forming bacteria were found in the course of the work and one of these, represented in the tables as W_{13} and 397 was isolated three times from sea-water collected at Ilwaco, Wash., Haines, Alaska, and Lynn Canal, Alaska. It was also isolated once from decomposing salmon. Although this organism appears to be widely distributed in the sea-water of that region it was not common in the decomposing salmon and was not found at all in the various parts of the canneries. When grown in pure culture this spore-former seems to have no effect on fish.

Cultures I and 420_a are orange-colored organisms identical in morphology and cultural reactions isolated once from salmon and once from a cannery at Haines. It was not found in the water samples examined. An orange-colored organism, represented by 401 and 443, was isolated once from salmon and twice from canneries at Sitka and Chatham, Alaska.

Cultures 400 and 471, are identical and were isolated once from salmon and once from a cannery at Yes Bay, Alaska.

In considering the sources of these various cultures, it may be borne in mind that all the isolations from salmon were made in Oregon and the fact that some cultures isolated there are identical with those obtained in Alaska indicates that such organisms have a wide distribution.

The frequency with which some bacteria are found in the cultures from decomposing salmon, when considered in connection with their decomposing action on fish, indicates that these organisms play an important part in the decomposition of the salmon. As explained in a previous report (Hunter, 1921) the salmon cultures studied in this investigation were obtained on successive days from salmon which were held under known conditions. The predominance of *Ps. fluorescens* throughout the viscera and the muscular tissue of the decomposed salmon leads to the conclusion that this organism is an important factor in the decomposition of the salmon. Two other organisms appear to play an important part in the decomposition of the salmon, namely, *Bact. cloacae* and the flesh-colored organism designated as No. 360. These organisms appear repeatedly on the plates and cultures made from decomposing salmon and when grown in fish broth in pure culture they produce very foul odors. As stated before, the other bacteria present may be regarded as accessory in the decomposition of the salmon but from this investigation it seems evident that *Ps. fluorescens*, *Bact. cloacae* and the flesh-colored organism (360) are of greater importance than any of the others.

This investigation has not shown that there are any different bacteria introduced by the use of the pew, or single-tine fork, than are to be found in the sea-water or in the salmon as it comes from the sea-water.

In obtaining cultures from canneries particular attention was paid to the exact location within the cannery from which the material was collected. This was done to determine whether there was any chance of contamination of the canned fish with spore-formers before cooking and also to determine whether there was a bacterial flora peculiar to the cannery or whether

the bacteria found within the cannery were those which came from the salmon and the sea-water. The investigation has shown that many of the organisms collected are generally distributed throughout the cannery and are not restricted to any particular part. In the case of such cultures as 430, 456 and 460, which were found eight, nine and seven times respectively, the organisms were isolated all the way along the canning line from the butchering table to the retorts. Since the location within the cannery, from which the culture was obtained, has no particular significance in a report of this kind, no note of it has been made in the tables. It is thought sufficient to give simply the geographical location from which the culture was obtained.

Of the 79 cultures reported here only 39 or about 49 per cent were found to have a common source such as sea-water and salmon, sea-water and the cannery or salmon and the cannery. If a larger number of cultures had been collected from a large number of sources throughout the salmon canning region, it is very probable that this percentage of correlation would be increased. Of the 15 cultures from sea-water there are only three (W_1, W_{1a}, and W_{9b}) which were not also found in salmon, in the cannery or in both salmon and the cannery. Fifteen cultures from salmon are identical with cultures from other sources and 14 cultures were isolated from salmon and not isolated from sea-water or from the canneries. Of the 35 cultures reported from canneries only 12 were obtained from other sources. In considering the comparatively small number of cultures collected (316) over such a large area it is not surprising that not more than 49 per cent of them were isolated from the three sources. There are only four cultures in the collection from sea-water (W_1, W_{1a}, W_{7b} and W_{9b}) which were not also found in decomposing salmon and it seems probable that if the number of cultures from salmon were larger it would include these four organisms. On the other hand it is also probable that, if it had been possible to collect a larger number of water samples and, hence, a larger number of cultures from water, very many of the 14 cultures from salmon, which it was impossible to correlate with any other source, would have been included in the sea-water flora. This is particularly true of such organisms as *Bact. coli*

and *Bact. communior*. It is apparent that, just as suggested previously, the bacteria causing decomposition in salmon are those forms the natural habitat of which is the sea-water from which the salmon are taken.

The correlation between the flora of the Alaskan canneries and the flora of the sea-water and the salmon is not as clear. Only about 34 per cent of the cultures from the canneries can be traced to another source and this leaves the source of 66 per cent of these cultures unexplained. There is the same probability existing here, however, that, if the number of sea-water cultures could have been increased the number of cannery organisms correlating with them might also have been increased. From this investigation, the outstanding fact about the bacterial flora of the Alaskan canneries is that it consists mainly of asporogenous, non-fermenting bacteria which appear to have very little effect on the decomposition of the salmon. This confirms the statement made in a previous report (Hunter, 1920) that the organisms concerned in the decomposition of salmon are those forms which are brought with the salmon from the sea-water and that the decomposition is not due to bacteria which contaminate the salmon within the cannery.

SUMMARY

In studying the distribution of the bacteria concerned in the decomposition of salmon, 316 cultures were collected from sea-water, from decomposing salmon and from salmon canneries throughout southeastern Alaska. By checking the duplicates this number was reduced to 85 cultures, one of which was an Actinomyces, one a pink yeast and four white yeasts. Of the remaining 79 cultures 72 were rod-shaped organisms without spores, 3 were spore-forming rods and 4 were streptococci. The morphology and cultural reactions of these 79 cultures are given.

While no attempt has been made to specifically identify many of the cultures, 6 have been identified as *Bact. cloacae*, 3 as *Bact. aerogenes*, 2 as *Bact. coli*, one as *Bact. communior* and 4 as *Ps. fluorescens*. The majority of the bacteria collected apparently belong to a large group of non-fermenting soil and water bacteria. These bacteria are similar to those mentioned by Jordan

(1903) in his report on the kinds of bacteria isolated from river water and are included in his Groups VIII to XIII inclusive. Conn (1917) stated that slowly liquefying or non-liquefying, non-spore-forming short rods such as these make up from 40 to 75 per cent of the organisms developing on aerobic plates inoculated with soil.

The results of this investigation indicate that *Ps. fluorescens*, *Bact. cloacae* and an unidentified flesh-colored organism play an important part in the decomposition of the salmon.

In determining the correlation between the bacteria from the three sources, it has been found that 80 per cent of the bacteria collected from sea-water are also found in decomposing salmon, in the canneries, or in both. Approximately 52 per cent of the salmon cultures were found elsewhere and about 34 per cent of the cultures from the Alaskan canneries were obtained from other sources.

The results of this investigation confirm the statement previously made (Hunter, 1920) that the bacteria concerned in the decomposition of salmon are those forms the natural habitat of which is the sea-water from which the salmon are taken and that the decomposition of salmon is not due to bacteria which contaminate the salmon within the cannery.

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VIABILITY OF THE COLON-TYPHOID GROUP IN CARBONATED WATER AND CARBONATED BEVERAGES

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The destructive effect of carbon dioxide on various microörganisms and the value of carbonation for the preservation of foods and beverages have claimed the interest of a number of workers since the first days of bacteriology. As early as 1885, Leone reported the examination of several commercial mineral waters which were under a slight pressure of CO_2. The number of microörganisms found to be present was always low. He also observed that after passing CO_2 gas through a drinking water the total count rapidly diminished.

Somewhat later than this a number of investigations were made of the destructive effect of CO_2 under relatively high pressures. Schaffer and Freudenreich (1891–1892), after studying the effect of pressures of 40 to 50 atmospheres of CO_2 combined with an increase of temperature, conclude that CO_2 has only a feeble bactericidal action. Sabrazès and Bazin (1893) found that cultures of *Bact. typhosum*, *Bact. coli*, *Staphylococcus aureus*, and the anthrax bacillus were able to develop after exposure to 60 to 70 atmospheres of CO_2 for several hours. These results are contradicted by D'Arsonval and Charrin (1893) who report that CO_2 under a pressure of 50 atmospheres sterilized cultures of *Ps. pyocyanea* in from six to twenty-four hours. Recently, Larson, Hartzell, and Diehl (1918), in a study of the effect of pressures upon bacteria, found that CO_2 under a pressure of 50 atmospheres would destroy *Bact. typhosum*, *Bact. coli*, *Mycobact. tuberculosis*, *Ps. pyocyanea*, staphylococci, strep-

tococci, and pneumococci, in a period of time ranging from one and one-half to two and one-half hours. Yeast cells were unaffected after an exposure of forty-eight hours.

Since these pressures are many times greater than those to which the ordinary carbonated beverages are subjected, there is the possibility that certain organisms may retain their vitality for a longer period. Several reports of the examination of carbonated beverages purchased in the open market have shown that occasionally there are encountered considerable numbers of microörganisms, including those indicative of pollution. Allen, LaBach, Pinnell, and Brown (1915) report a sanitary survey of the "soft drink" industry of Kentucky. Although carbonation was found to cause a distinct reduction in the numbers present, occasional high counts and the presence of *Bact. coli* were reported. Stokes (1920) recently examined a great variety of "soft drinks" and noted the frequent presence of *Bact. coli* in 10 cc. and 1 cc. quantities, with an occasional occurrence in 0.1 cc. The plate counts exhibited great variation, and while the majority of samples yielded counts of less than 100 per cubic centimeter, a few showed surprisingly high numbers. Gershenfeld (1920) reports similar results. Young and Sherwood (1911) have reported an experiment in which they determined the viability of *Bact. typhosum Bact. coli*, and *Erythrob. prodigiosus* in carbonated water to which lemon syrup had been added. Although the typhoid bacillus showed a considerable reduction in numbers after four hours exposure, a few viable cells were found after ten days. *Bact. coli* and *Erythrob. prodigiosus* were found to be somewhat more resistant than *Bact. typhosum*.

In the present investigation chief emphasis has been placed upon the colon-typhoid group for the purpose of determining the length of time one may expect the various members of this group to withstand the environment of the different types of commercial carbonated beverages.

The following organisms have been employed: *Bact. coli* (fecal origin), *Bact. paratyphosum* B, and *Bact. typhosum*. Also, as a matter of interest, two common spore forms were included; *B. mesentericus*, and a putrefactive anaerobe of the *Clostridium sporogenes* group.

The beverages were prepared and carbonated in the 7-ounce bottles commonly used in the industry. Since they were prepared as nearly as possible under commercial conditions and no effort was made to sterilize the various ingredients, control examinations of the product were made previous to experimental inoculation to determine the absence of the particular type of organism used in the investigation. In no instance was any difficulty of this kind encountered. Throughout the work commercial CO_2 was used for carbonation. As a test for any impurities in the carbon dioxide which might affect the death-rate of the organisms, tap water was carbonated as usual, then heated in the Arnold sterilizer for a short period to expel the CO_2 and finally the death-rate of *Bact. coli* in this water was compared to that in parallel samples of the original tap water. No discrepancies other than those which might be attributed to experimental variation were observed.

Small amounts of a suspension of the various test organisms in sterile tap water were used for inoculation. This was accomplished in one of the two following ways. The first method consisted of adding equal amounts of bacterial suspension to each bottle just before carbonation. In the second method the samples were prepared, bottled, carbonated, and capped as usual. They were then stored at 1°C. for several days until used, when the bottles were re-opened and inoculated. If opened while still cool, there was little loss of CO_2 gas. The first method was used for most of the experiments with *Bact. coli*. The second method was necessary when working with *Bact. typhosum* and *Bact. paratyphosum* B since by the first method there is more or less spattering of the material during the process of carbonation.

Immediately after inoculation and at definite intervals thereafter plate counts were made. To prevent the considerable loss of CO_2 upon repeated opening of the same bottles, especially those held at room temperature, a number of bottles were inoculated with equal amounts of bacterial suspension and, at each time interval, different sets of two were opened and samples withdrawn for plating. When the numbers had become so

reduced as to give negative results upon plating 1 cc. quantities, larger amounts, 5 cc. and 10 cc., were introduced into broth to determine, insofar as possible, the final disappearance of the organisms in question. This was done by streaking Endo plates from the broth cultures, fishing any typical colonies, and finally applying the usual methods used for the identification of the various members of this group of organisms.

EXPERIMENTAL

Since temperature may be expected to exert a marked influence upon the death-rate, experimental samples were held at two different temperatures, namely, in cold storage at 1°C. and at room temperature, 19° to 23°C. Tables 1 and 2 present data showing the viability of *Bact. coli* in carbonated water at several different pressures and also, for purposes of comparison, in plain tap water. It is evident that carbonation causes a speedy destruction of the colon bacillus and that this effect is dependent upon the temperature at which the samples are held, being much more pronounced at room temperature than at 1°C. Furthermore, the different degrees of pressure of CO_2 mentioned in these tables apparently exerted little or no influence upon viability, for the organisms were killed as speedily in water saturated with CO_2 (at both 20°C. and 1°C.), but under no excess pressure, as they were in the carbonated samples under pressures of 28 and 41 pounds per square inch. In fact, where the pressure was released the plate counts frequently were less than those of the samples held under pressure (table 2), a phenomenon which was regularly observed upon several repetitions of the experiment.

To gain an idea of the hydrogen-ion concentration of carbonated water the indicators brom-phenol-blue and methyl-red were added to different bottles which were then filled with carbonated water at these several pressures. In this way the value was roughly determined as pH 4.0–4.4. Release of the pressure, as indicated in table 2, was followed by very little, if any, immediate change in the hydrogen-ion concentration when meas-

ured in this way. When, however, such samples are held for a period of one or two weeks at 19° to 20°C. there is a gradual escape of CO_2 gas as evidenced by a decrease in the hydrogen-ion concentration. It is believed that under the conditions of our experiments the acidity of the dissociated carbonic acid is

TABLE 1

Showing the comparative viability of Bact. coli in carbonated tap water under pressure and in plain (non-carbonated) tap water

	TIME INTERVAL	CARBONATED TAP WATER PRESSURE 41 POUNDS PER SQUARE INCH (2.78 ATMOSPHERES) AT 18°C.		CONTROLS, PLAIN TAP WATER	
Held at room temperature (20-21°C.)	At once	181,000*	156,000	204,000	190,000
	4 hours	79,000	80,000		
	24 hours	950	6,600	203,000	194,000
	4 days	1 cc. plate negative	26	34,200	18,000
	7 days	10 cc. 0†	10 cc. +	14,800	10,000
		5 cc. +	5 cc. 0		
		1 cc. 0			
	14 days	10 cc. 0	10 cc. 0	2,200	1,000
		5 cc. 0			
Held at 1°C.	At once	163,000	181,000	200,000	260,000
	24 hours	51,000			
	4 days	25,500	23,000	166,000	180,000
	7 days	2,700	5,400	190,000	Lost
	14 days	1 cc. plate negative	30	100,000	Lost
	26 days	5 cc. +	1 cc. +	17,000	Lost
		1 cc. 0	0.1 cc. +		

* Figures represent numbers of *Bact. coli* per centimeter.

† 0 indicates the absence, and + the presence, of *Bact. coli* as determined by transferring the specified amount of water (10, 5 or 1 cc.) to broth. This was done when the numbers had become so reduced as to give negative results upon plating 1 cc. quantities.

the main factor responsible for the death of the bacteria. Other factors, such as differences in osmotic pressure, may also play a part.

Several of the simpler carbonated beverages were used in the next experiments. It should be realized that certain acids—

usually citric, tartaric, phosphoric, or lactic—are added to some types of beverages and that these acids may affect the longevity of the organisms in question. A comparison of the viability of *Bact. coli* in a non-acid and in an acid-containing beverage is shown in table 3. It will be noted that the hydrogen-ion concentration of the latter is considerably greater than that of the former. The colon bacillus is killed much more speedily in the acid-containing beverage, the effect being especially marked at the higher temperature. Apparently the rapid de-

TABLE 2

Viability of Bact. coli in carbonated tap water under pressure and with pressure released

	TIME INTERVAL	PRESSURE 28 POUNDS PER SQUARE INCH (1.9 ATMOSPHERES) AT 24°C.		EXCESS CO_2 ALLOWED TO ESCAPE AT THE RESPECTIVE TEMPERATURES BEFORE INOCULATION	
Held at room temperature (22–24°C.)	At once	440,000	360,000	440,000	500,000
	24 hours	1,500	1,360	870	130
	3 days	13	18		36
	7 days	10 cc. +	10 cc. 0	10 cc. +	10 cc. +
		5 cc. 0		5 cc. 0	5 cc. 0
	18 days	10 cc. 0	10 cc. 0	10 cc. 0	10 cc. +
					5 cc. 0
Held at 1°C.	At once	500,000	390,000	300,000	540,000
	24 hours	34,000	14,700	8,200	10,300
	3 days	3,600	900	83	71
	7 days	100	88	1 cc. +	1 cc. +
	18 days	1 cc. +	1 cc. +	1 cc. +	1 cc. +

struction of *Bact. coli* in the acid-containing lemon soda is due mainly to the dissociation of the citric acid present, for in additional experiments in which this acid was omitted the death rate was found to be comparable to that of the non-acid vanilla soda. Also in this case the hydrogen-ion concentration had decreased from pH 3.0 to pH 4.0–4.4 by the omission of the citric acid.

Experiments with other acid-containing beverages have demonstrated that as the amount of acid is increased above that indicated in table 3, the more readily is the colon bacillus killed.

In one instance in which 0.156 per cent lactic acid (5 grains per 7-ounce bottle) was employed the numbers of *Bact. coli* dropped from several hundred thousand to several hundred per cc. in

TABLE 3

Showing the viability of Bact. coli in a non-acid and in an acid type of carbonated beverage

	TIME INTERVAL	VANILLA SODA (NON-ACID), PRESSURE 24 POUNDS (1.6 ATMOSPHERES) AT 21°C., pH 4.0-4.4		LEMON SODA (ACID TYPE), PRESSURE 19 POUNDS (1.3 ATMOSPHERES) AT 23.5°C., pH 3.0	
Held at room temperature (21–24°C.)	At once	132,000	136,000	245,000	160,000
	24 hrs.	20,900	32,700	900	200
	3 days			1 cc. negative	1 cc. negative
	4 days	140	20	10 cc. negative	10 cc. negative
	7 days	1 cc. positive	1 cc. positive		
	14 days	10 cc. negative	10 cc. negative		
Held at 1°C.	At once	140,000	127,000	225,000	215,000
	24 hrs.	68,000	46,000	115,000	144,000
	3 days			87,000	25,000
	4 days	17,200	13,500		
	7 days	11,100	11,200	6,800	4,900
	14 days	390	600	600	
	1 mo.	1 cc. positive	1 cc. positive	10 cc. negative	10 cc. negative
	2 mos.	10 cc. positive; 1 cc. positive	10 cc. positive; 1 cc. negative		

Composition of the above beverages:

Vanilla soda: 10 mgm. c.p. vanillin (0.0048 per cent) and 10 grams sucrose (4.8 per cent) per 7-ounce bottle (207 cc.).

Lemon soda: 0.5 cc. commercial lemon flavor, 3 grains citric acid (0.094 per cent), and 20 grams sucrose (9.6 per cent) per 7-ounce bottle.

Carbonated water added to make the finished beverage.

4 hours at 20°C. When plain non-carbonated tap water was substituted for the carbonated water in the acid beverages, the death-rate of *Bact. coli* remained practically the same. That is, in these instances the added acids are the chief causative agents

in the destruction of the colon bacillus, irrespective of the effect of carbon dioxide.[1]

Since it was found that *Bact. coli* is able to withstand carbonation for an appreciable period, the next step was to investigate the viability under similar conditions of several of the pathogenic members of the colon-typhoid group. In these experiments *Bact. paratyphosum* B and *Bact. typhosum* were used. It was at once apparent that both of these types are considerably less resistant to the destructive effect of CO_2 than is the colon bacillus. Table 4 presents results which are characteristic of a number of similar experiments. One point of particular interest is the persistence, at 1°C., of the last few surviving organisms. These were too few in number to be estimated by plating and their presence could be detected only by the cultivation of 10 cc. amounts in glucose broth. Additional experiments with acid-containing beverages have shown that in these the typhoid bacillus is killed almost instantly. Thus in a carbonated lemon soda containing 0.156 per cent lactic acid, *Bact. typhosum* decreased in numbers from an initial inoculum of 27,400 per cubic centimeter to 10 per cubic centimeter within one hour and after two hours its presence could not be detected.

It should be emphasized that throughout all of the foregoing experiments the water used for carbonation and for preparation of the various beverages was an ordinary city supply of low mineral content. Under certain conditions, as for example in carbonated water of high mineral content, it is possible that non-spore-forming organisms may remain alive for longer periods than those herein reported. This possible influence of certain inorganic salts upon the viability of microörganisms in a carbonated environment has not been studied in the present investigation.

[1] The usual methods of bacteriological analysis could not be applied when larger quantities of the highly acid beverages were to be examined. It was found that sufficient amounts of acid were carried over to the culture medium to cause a distinct increase in the H-ion concentration, sufficient, indeed, to effect a retardation or even complete inhibition of growth. By the use of larger quantities of broth in flasks, instead of the usual amounts ordinarily contained in test tubes, this difficulty was largely overcome.

TABLE 4

Comparative viability of Bact. typhosum in carbonated and in plain (non-carbonated) tap water

TABLE 4

Comparative viability of Bact. typhosum in carbonated and in plain (non-carbonated) tap water

TIME INTERVAL	CARBONATED TAP WATER, PRESSURE 25 POUNDS (1.7 ATMOSPHERES) AT 24°C.				CONTROL, PLAIN TAP WATER	
	Held at room temperature (22-24°C.)		Held at 1°C.		Held at room temperature (22-24°C.)	
At once	45,000	36,500	44,400	27,000	42,000	63,000
24 hours	1 cc. plate negative	1 cc. plate negative	30	1 cc. plate negative	41,000	22,000
48 hours	10 cc. negative	10 cc. negative	10 cc. positive	10 cc. positive	27,000	12,000
4 days			10 cc. positive	10 cc. positive		
7 days			10 cc. negative	10 cc. negative		

Comparative viability of Bact. typhosum and Bact. paratyphosum B. in vanilla soda

	PRESSURE 23 POUNDS (1.56 ATMOSPHERES) AT 24°C.							
	B. typhosum				B. paratyphosum B			
	Held at room temperature (21-24°C.)		Held at 1°C.		Held at room temperature (23-25°C.)		Held at 1°C.	
At once	175,000	166,000	237,000	144,000	170,000	116,000	194,000	200,000
4 hours					16,200	800		
24 hours	40	270	10,300	21,600	1 cc. plate negative	3	2,600	7,300
48 hours	1 cc. plate negative	1 cc. plate negative	310	170	10 cc. pos. 1 cc. neg.	10 cc. pos. 1 cc. neg.	430	860
3 days	10 cc. plate negative	10 cc. plate negative	10 cc. pos. 1 cc. neg.	10 cc. pos. 1 cc. neg.	10 cc. negative	10 cc. negative	10	15
4 days	10 cc. negative	10 cc. negative	10 cc. positive	10 cc. positive	10 cc. negative	10 cc. negative	10 cc. positive	10 cc. positive
6 days			10 cc. positive	10 cc. positive				
7 days							10 cc. positive	10 cc. positive
10 days			10 cc. negative	10 cc. negative			10 cc. negative	10 cc. positive
14 days							10 cc. negative	10 cc. negative

The resistance of spores to the conditions described in this paper is of some interest when compared to that of *Bact. coli* and *Bact. typhosum*. The spores of both *B. mesentericus* and *Clost. sporogenes* were found to be quite resistant, for after one month in carbonated water no reduction in numbers could be detected. In one experiment the spores of *B. mesentericus* survived in a citric acid beverage (pH 3.0) for one month with little, if any, diminution in numbers.

It must be stated emphatically that the results obtained in this investigation do not warrant the conclusion that water of a low sanitary quality can be used by the industry in the preparation of carbonated beverages, or that carbonation can be relied upon to destroy evidence of pollution. In many instances, particularly during the summer months, beverages are consumed within a few hours after their preparation and it is obvious that under these conditions pathogenic organisms, if originally present in the water, may survive carbonation and reach the consumer.

SUMMARY

Under the conditions of these experiments carbonation exerts a distinctly harmful effect upon the members of the colon-typhoid group and their period of viability in carbonated water is much shorter than that in plain tap water. The destructive effect of the CO_2 is especially marked at room temperature, 19° to 23°C., and less so at 1°C.

In a "non-acid" beverage, the organisms may persist for a slightly longer period than in carbonated water. In beverages containing 0.094 per cent or greater amounts of citric or lactic acids, the death-rate is very rapid and is apparently due to the effect of these acids, irrespective of the CO_2.

Bact. typhosum and *Bact. paratyphosum* B are more readily destroyed by CO_2 than is *Bact. coli*.

The spore forms of a common aerobe, *B. mesentericus*, and of a common anaerobe, *Clost. sporogenes*, were found to be quite resistant to carbonation, surviving one month at room temperature with no apparent diminution in numbers.

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A BINOCULAR MICROSCOPE ARRANGED FOR THE STUDY OF COLONIES OF BACTERIA

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The binocular microscope arranged as described below has been used in this laboratory for several years with so much satisfaction and profit in the isolation of bacteria that a description of such a simple piece of apparatus may not be out of place.

Every one who has examined colonies of bacteria or other organisms on culture media by the use of direct illumination from below has experienced more or less difficulty. The medium is frequently too opaque to admit sufficient light, especially in the case of media containing blood; some very small colonies have the same refractive index, or so nearly the same refractive index, as their medium that observation may be very difficult even on transparent media. These difficulties are obviated and observation greatly facilitated by viewing the colonies in light reflected from the surface of the culture as is usually done in using a hand lens for this purpose. To make such observations with higher magnification and greater ease we arranged a binocular microscope with magnifications of ten and twenty diameters in the following manner. The tube and focusing apparatus was removed from the stage and base and attached to an independent support so that its optical axis was at an angle of 45° with the stage, though so arranged that the angle might be altered as desired (*A*, fig. 1). A small arc with a condensing lens was supported so as to project a beam of light at an angle of 45° with the stage and at right angles to the optical axis of the microscope. As this gave rather more light than was necessary a 75-watt "daylight" lamp was supported about

15 cm. above the stage and surrounded, except for a slit 2 cm. wide, with an opaque reflecting screen in such a position that a beam of light was projected to the stage at right angles to the optical axis of the microscope (*B*, fig. 1). A solid black stage provided support for Petri dish cultures or a groved block placed on it provided for the support of tube cultures so as to bring the surface of the media parallel with the stage.

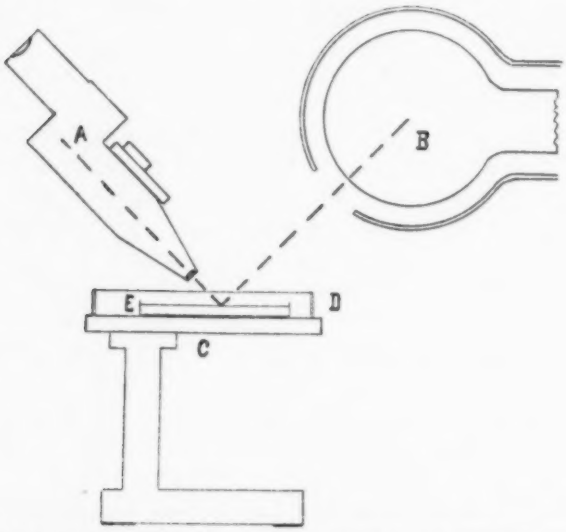


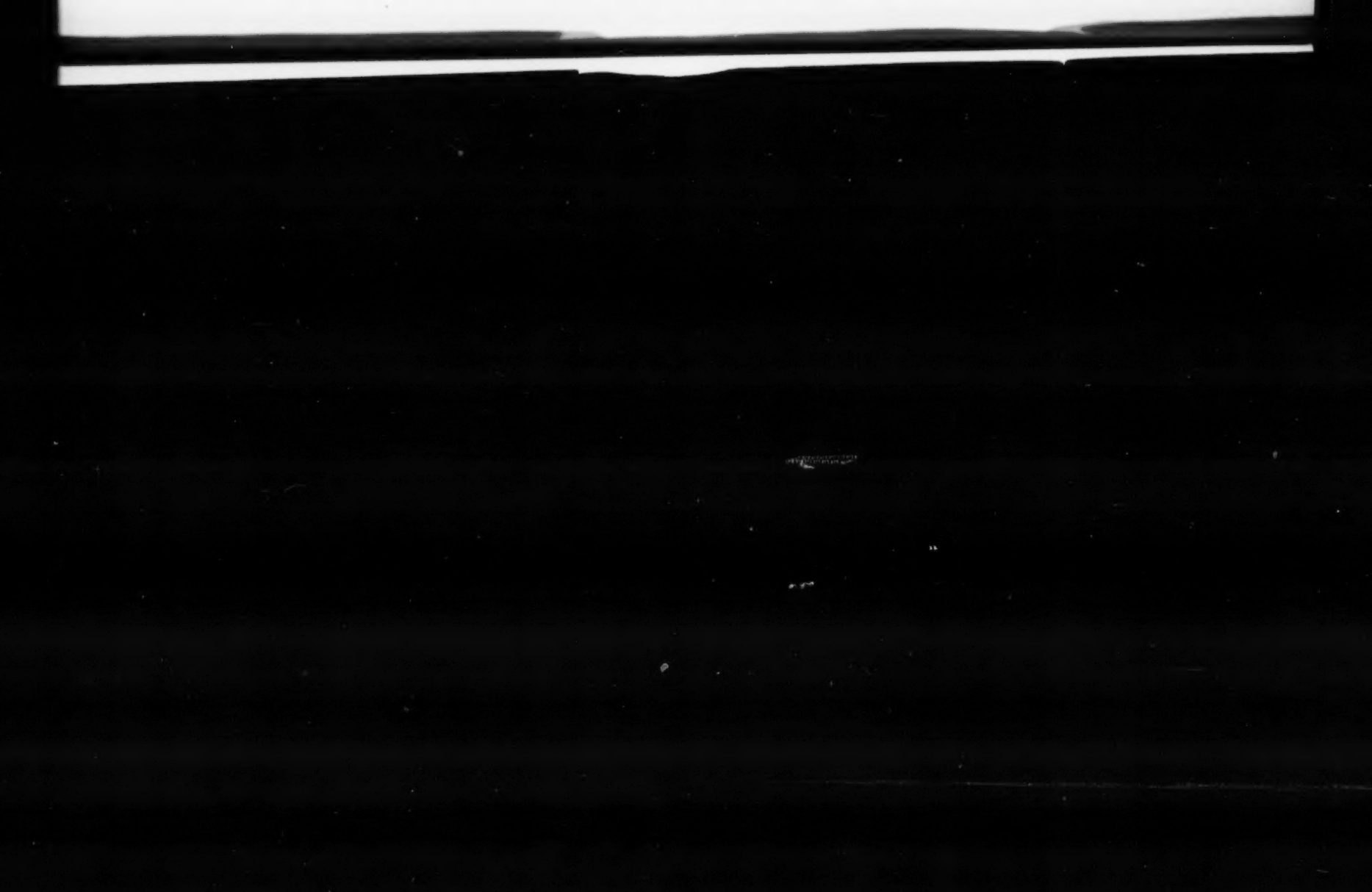
FIG. 1. A DIAGRAM OF A BINOCULAR MICROSCOPE ARRANGED FOR THE STUDY OF COLONIES ON THE SURFACE OF MEDIA

A, Binocular microscope tube and focusing apparatus supported on a base independent of the stage and at an angle of 45° with the stage; *B*, "daylight" 75 watt light surrounded by a screen except for a slit; *C*, solid black stage and base; *D*, glass screen to protect open plate cultures; *E*, Petri dish culture.

The focal distance of the binocular is sufficient to permit of the examination of tube cultures or Petri dish cultures with the cover left on. For the fishing of colonies from cultures which were to be further incubated a plain glass was supported 15 mm. above the stage by strips along three sides leaving the right

hand side open. An uncovered plate culture might then be slipped under this glass screen with very little danger of dust or breath contamination and at the same time permit the fishing of colonies, under microscopic observation, with a platinum wire. The most satisfactory platinum wires for this purpose were drawn out in the flame to a fine point which was then turned at a right angle about 1 mm. from the tip.

The chief value of the apparatus in this laboratory has been in the isolation of organisms producing very small colonies from material containing large numbers of other species, i.e., the isolation of *H. influenzæ* from sputums. It is a very easy matter to fish the smallest *H. influenzæ* or similar colonies of other species, or to remove half of such a colony and smear for microscopic observation and later to use the remaining half colony for the inoculation of subcultures.



AN INVESTIGATION OF AMERICAN STAINS

REPORT OF COMMITTEE ON BACTERIOLOGICAL TECHNIC

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Early in 1920 the Committee on Bacteriological Technic was asked to look up the matter of biological stains at present available in America and to see what could be done toward standardizing them. At present it is difficult, if not impossible, to obtain the Grübler stains, and the American products are known to be variable. The impression has even been common that American dyes are generally unsatisfactory for staining.

Upon looking into the matter, the committee has found that many American stains are as good or even better than the old Grübler stains, except for certain special uses which their producers did not have in mind when preparing their products. Certain American producers of biological stains are trying very hard to put on the market an entirely satisfactory line of goods. The difficulty comes from the fact that the field is a small one, and so much competition has arisen that no one can make a satisfactory profit. The danger is that soon the American producers will all be driven out of the business and importation of stains will again be necessary. We do not desire to be dependent upon foreign production in this line, because of the great importance of stains in public health work and the possibility of another national emergency when importation will be impossible. To relieve the situation, therefore, some one manufacturer must be given enough support to make the business profitable for him. This means standardizing on one line of stains, either all produced by the same house, or if produced by different concerns, each particular stain coming from one manufacturer only.

[1] For the numerous collaborators in this work, see list at end of the report.

In order to survey the field it seemed necessary to test products from as many sources as possible. As the whole field is a large one, the attention of the committee at first was given to but three stains or groups of stains; fuchsin, methylen blue, and gentian violet. About thirty members of the Society,—to whom much credit is due,—volunteered to assist. Their names are given at the end of this paper; and without their hearty cooperation, the work would have been impossible. Our study is not complete yet, but a fairly satisfactory survey of the field has been made, so far as concerns fuchsin and methylen blue. The gentian violet situation is more complicated and further investigation is necessary.

Upon looking into the commercial situation, it proved necessary to obtain samples of the dyes from two different classes of dealers: the basic dye manufacturers and the so-called "manufacturers and standardizers" of biological stains. The former group are the manufacturers of general textile dyes, and embrace such concerns as the National Anilin Company, the duPont Company, the Calco Chemical Company, and Dicks, David and Company. The latter group generally buy their dyes from the former, make certain tests of them and put them on the market as biological stains, with or without modification; their chief function is standardization rather than manufacture. Representatives of the latter group are the Providence Chemical Laboratories, the H. S. Laboratories, the Coleman and Bell Company (formerly the National Stain and Reagent Company), and the Heyl Chemical Company. Samples from these various concerns were obtained through a jobber who had consented to coöperate in the work. This was done so that no concern would know the reason for which the samples were bought. To make unbiased opinions still more certain, the samples were distributed among the various investigators by number only, without reference to the names of the dealers.

Throughout this work, the committee has been in correspondence with all of the second group of dealers mentioned above, and with the Calco Chemical Company as well. There has seemed no purpose in getting in touch with the other basic

manufacturers, as the production of biological stains is a very small part of their business and the results of this work mean little to them. In general, hearty coöperation has been secured. All but two of the dealers have told us to feel perfectly free to publish the results. As these two are merely distributors of scientific supplies, and as it is difficult to tell the original source of the samples obtained from them or to learn whether their present supply is still the same, their names are suppressed here, using instead the designations A and B. For similar reasons the name of a third house of like character is suppressed, although in this case the committee was not asked to do so.

The list of dealers (other than these three) whose products have been examined is as follows. In this list the addresses are given for those firms who deal specially in biological stains.

Calco Chemical Company, 136 Liberty Street, New York City
Coleman and Bell Company, Norwood, Ohio
Dicks, David and Company
E. I. duPont de Nemours Company
Geigy Chemical Company
Goldin Biological Laboratories, Providence, R. I.
Harmer Laboratories Company, Lansdowne, Pa.
Heyl Laboratories, 437 Baretto Street, New York City
Holland Anilin Company
H. S. Laboratories, 6005 Girard Avenue, Philadelphia
H. Kohnstam and Company
Mallinckrodt Chemical Company
Merck and Company
National Anilin Company
Newport Chemical Works
Providence Chemical Laboratories, 51 Empire Street, Providence, R. I.
Williamsburg Chemical Company

Very few tests were made with the products of the Heyl Laboratories. This was merely because the Heyl products are sold under certain restrictions that make it difficult for jobbers to buy them. We could have obtained all the Heyl stains we

wanted direct from the company; but for the sake of fairness we did not want to buy this one brand of stains direct, while picking up the others on the open market. The jobber through whom we dealt had such delays in getting the Heyl samples that they were not ready at the time of our main tests. It might further be remarked that Mr. Heyl himelf says that his crude dyes are generally obtained from abroad, as he does not believe the American products satisfactory.

One of the troublesome factors in the stain situation is due to confusion in nomenclature. It may happen that the same name is given to more than one product or that the same product is called by several names. Accordingly it seems worth while to discuss these three stains from the standpoint of their chemical composition and to list their synonyms. In the list of synonyms in each case, preferred names are given in bold-faced type.

FUCHSIN (BASIC)

Synonyms

Rosanilin
Diamond fuchsin
Magenta
Rubin
Anilin red

The formula of fuchsin, or rosanilin, is $(NH_2.C_6H_4)_2:C:C_6H_4:NH$. Being a basic dye, this is ordinarily combined with an acid, and the dye with which we are familiar is the chloride $(NH_2\cdot C_6H_4)_2:C:C_6H_4:NH:HCl$. Fuchsin proves the easiest to obtain in a satisfactory state of purity of any of the dyes we have so far investigated.

There are three chief uses of fuchsin in bacteriological work: general bacterial staining; staining for acid-fast qualities; and use in the endo medium for colon-typhoid differentiation. A satisfactory stain should give good results for all three purposes. In the present investigation, the acid-fast test has been most frequently used, but enough tests have been made for the other purposes to indicate the general utility of the different samples. Only two investigators have used the stains in the endo medium,

both of them using different technics; although one of these two (Castleman) made his tests on two different occasions, with a different technic each time.

Results were reported in two different ways. Some grouped the samples in three or four categories which it has proved possible to denote by four terms: excellent (E), good (G), fair (F), and unsatisfactory (U). Others listed the samples in the order of their excellence, in which case instead of placing them in these four categories, they have been numbered 1, 2, 3, etc., the low numbers indicating the best samples. These two sets of symbols are used in Table 1, where the results of the different investigators with the different samples are listed.

It will be seen that there is some variation in the findings of the different investigators, although not as much as in the case of the other two dyes, reported below. When fuchsin was used as a stain it proved difficult to pick out any one sample or any two or three samples that were superior to the others. To make the comparison more definite, each of the four classes was given a numerical symbol, these numbers averaged, and the average converted back into the class symbol again, using the grades: E, E−, G+, G, G−, etc. By glancing at this average grade, it will be seen that the sample from the Providence Chemical Company ranks G+, none of the others ranking better than G. All but three of the others rank G.

The samples are rearranged in table 2 in the order of excellence, according to their average grade, this same table listing the number of times each sample was found excellent and the number of times found unsatisfactory. Only three samples were reported unsatisfactory by any one, namely those from dealers A, B, and C, each being so reported by two different investigators. These three were the only samples to have an average grade lower than G, and yet two of them were reported as *good* or *excellent* by some investigator. There seems no reason to feel that any of the samples, with the exception of these three, could not be successfully substituted for the Grübler product. The superiority of the Providence sample is so slight as to be of little significance.

TABLE 1

Reports of the individual investigators on the samples of fuchsin

	AS STAIN INVESTIGATOR AND TEST USED											AVERAGE GRADE AS STAIN	AS INDICATOR ON INVESTIGATOR AND TEST USED				AVERAGE GRADE AS INDICATOR
	Castleman, Ziehl stain	Castleman, Gram counter-stain	Boyd, Ziehl stain	Hill, Ziehl stain	Burnett, Ziehl stain	Meyers, Ziehl stain	Meyers, General stain	Craig, Ziehl stain	Roos, Ziehl stain	Wade, Ziehl stain	Pickens, General stain		Wade, Endo medium	Meyers, Endo medium	Castleman, first test, Endo medium	Castleman, second test, Endo medium	
Calco	12	9	G	3	G	G		G	G	E	G	G	F	E	U	G	G−
Dealer A	6	11	G	1	G	U	U			G	U	F	U	U	U	U	U
Harmer	7	3	G		G	G	G			E	F	G	E	G	U	F	G−
National Anilin	10	2	G	2	G	G	E			G	F	G	G	G	U	G	G−
Newport	2	8	G	7	G	G	E			F	F	G	F	E	U	G	G−
Providence	3	4	G		G	E	E	G	G	G	E	G+	G	E	U	F	G−
Coleman and Bell	5	1	G	5	G	G	E	G	G	F	F	G	E	G	G	G	G+
Goldin	1	5	G		G		G	G	G	F	G	G		G	U	G	G−
H. S.	8	7	G	6	G	G	G	G	E	E	F	G	F	G	U	F	F
Dealer B	11	10	G	4	G	E	G	E	G	U	U	G−	F	E	U	G	G−
Dealer C	9	12	G	9	U	U	U			F		F−	U	U	U	U	U
Dicks, David and Company	4	6	G	8	G	G	G			G	E	G	G	G	U	G	G−

When used in the endo medium, the samples were reported quite differently. The result plainly depends on the technic, some methods calling for too concentrated a solution of the dye to be decolorized by the amount of sulfite ordinarily used. The following formulae were used:

Meyers: 0.5 cc. of 10 per cent alcoholic fuchsin added to 10 cc. of 2.5 per cent sodium sulfite solution. This quantity added to 100 cc. of the agar. (Standard method of American Public Health Association.)

TABLE 2

Summary of reports on fuchsin; samples arranged in order of excellence

SAMPLE	AS STAIN					IN THE ENDO MEDIUM
	Number of tests	Number of times excellent	Number of times unsatisfactory	Average grade	Conclusions	
Providence	10	3	0	G+	Very good	Good
Calco	10	1	0	G	Good	Good
Coleman and Bell	11	2	0	G	Good	Excellent
Dicks, David	9	0	0	G	Good	Good
Goldin	10	1	0	G	Good	Good
Harmer	8	2	0	G	Good	Good
H. S. Laboratories	11	2	0	G	Good	Good
National Anilin	9	2	0	G	Good	Good
Newport	9	2	0	G	Good	Good
Dealer B	11	2	2	G−	Fair to good	Good
Dealer A	9	1	3	F+	Fair to poor	Unsatisfactory
Dealer C	8	0	3	F−	Unsatisfactory	Unsatisfactory

Castleman, first test: 1 cc. of saturated alcoholic fuchsin added to enough 10 per cent sodium sulfite solution to decolorize. One cubic centimeter added to 100 cc. of agar.

Castleman, second test: 0.5 cc. of 1 per cent alcoholic fuchsin added to 1.5 cc. of 2.5 per cent sodium sulfite solution. One cubic centimeter added to 100 cc. of the agar.

The only sample giving universally satisfactory results in these tests is that of the Coleman and Bell Company. This finding has been corroborated by other results not included in the present series of tests. By using the proper technic, however, i.e., either the first or the third formula above, good results

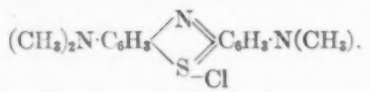
were obtained with all the samples except those from dealers A and C. These two, it will be noticed, are the two poorest samples when used as stains. It has been found that even the Grübler product shows up no better in such tests, the Coleman and Bell sample being even more satisfactory than the German fuchsin.

For these reasons our present recommendation in regard to fuchsin is in favor of the product of the Coleman and Bell Company. Although this does not show up quite as well in the staining tests as that of the Providence Chemical Company its rank is good, and its superiority as an indicator in the endo medium puts it almost in a class by itself. It is plain, however, that good results can be obtained with any of the samples except the three that stand lowest in table 2.

METHYLEN BLUE

Synonyms
Ethylene blue
Swiss blue

The chief confusion in regard to methylen blue lies in the fact that there are really two dyes on the market, one a pure chloride of methylen blue, the other a double salt, zinc and methylen blue chloride. The pure chloride has the formula:



The methylen blues which are not zinc-free are generally known as "methylen blue" or "methylen blue for bacilli." The free chloride, on the other hand, is specified in one of the following manners:

Methylen blue, medicinal (pure, zinc-free).
Methylen blue, U. S. P.
Methylen blue, BG.

The free chloride is soluble in both water and alcohol, the zinc salt is insoluble in alcohol.

Grübler's methylen blue for bacilli was apparently mostly zinc salt, this being the cheapest form of methylen blue available. It was not pure, however, and thanks, apparently, to the fact that small amounts of the zinc-free compound were present in it, it could be used in the Loeffler formula, which calls for 3 parts saturated alcoholic solution to 10 parts water. Unfortunately some of the preparations now on the market called "methylen blue for bacilli" are so nearly pure zinc salt as to be unsatisfactory for the Loeffler solution. On the other hand, some American stains labelled exactly the same are apparently nearly zinc-free, and are admirable for staining purposes, even in the Loeffler formula.

In the present series of tests, samples of both the zinc salt and the medicinal (zinc-free) dye were used. Fourteen investigators took part in the tests, and the samples were tested in several different ways. Four men, Healy, Hunter, Macy and Robertson, used the samples for staining dried milk smears according to the Breed method of examining milk for bacteria; Macy and Robertson used aqueous solutions of the stain samples, Healy and Hunter the Loeffler solution. Healy and Robertson also used their samples for staining ordinary bacterial cultures, and with the exception of Levine, all the other investigators used the stains for this purpose only, all employing the Loeffler solution, except Robertson who used a saturated aqueous solution. Levine tested the samples by using them in his eosine-methylen-blue medium for colon-aerogenes differentiation.

The test made by the writer was a special test made at the suggestion of Mr. Bell of the Coleman and Bell Company. Only those samples were selected for it which had already been found to give the best results. Three solutions of each were prepared: a 1 per cent aqueous, a 3:10 alcoholic aqueous (i.e., 3 parts saturated alcoholic solution to 10 parts distilled water), and a Loeffler solution (i.e., a 3:10 mixture using 0.0001 per cent NaOH instead of distilled water). Smears of a diphtheria culture were stained with each, allowing only about three seconds for staining and then washing under a tap. Mr. Bell suggested this as the most severe test he knew, and one which his methylen blue for bacilli was required to meet.

TABLE 3

Report of individual investigators on the samples of methylen blue

	AS STAIN INVESTIGATOR, SOLUTION AND MATERIAL STAINED															AVERAGE GRADE AS STAIN	INVESTIGATOR AND INDICATOR
	Healy, Loeffler, milk	Healy, Loeffler, B. coli	Macy, aqueous, milk	Leonard, Loeffler, cultures	McConnell, Loeffler, cultures	Voigt, modified Loeffler, cultures	Ritter, Loeffler, cultures	Hunter, Loeffler, milk	Heinemann, cultures	Lipman, Loeffler, cultures	Huntoon, Loeffler, cultures	Roos, Loeffler, cultures	Robertson, aqueous, cultures	Robertson, aqueous, milk	Conn, various B. diphth.		Levine, as indicator
Samples "for bacilli":																	
Calco	E	F		G		G	U	U	G				G	F		F+	G
Dicks, David				U	U			U	F		U	U	G	U		U	U
Dealer A	U	F	F				U	U					G	F		F−	E
Harmer				F		G	U				U	G	E	G		F+	U
Kohnstam	U	U	F		U			U		E			G	F		F	E
National Anilin	U	G	F			E			G+				G	F		G−	F
Holland Anilin			F	U				U		G			G	F		F	G
Providence					U	U			G	F			G	G		F	G
Geigy				U	U				F		U	G	G	F		F	U
Heyl														E	E		
Unknown Sample	U	U	U		E			U		F			F	U		F−	U
Coleman and Bell	U	E	F				E	G		G			E	E	E	E−	E−
H. S.				G		G	U		U				F	G		F+	U
Medicinal samples:																	
Calco			E		E	G				E			G	E	E	E−	G
Dealer A				F	U	G			U	G			G	F		F	E

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TABLE 4

Summary of reports on methylen blue; samples arranged in order of excellence

SAMPLE	AS STAIN					IN THE LEVINE MEDIUM
	Number of tests	Number of times excellent	Number of times unsatisfactory	Average grade	Conclusions	
For bacilli:						
Coleman and Bell	9	5	1	E−	Very good	Excellent
Heyl	2	2	0		Very good	Not tested
National Anilin	7	1	1	G−	Fair to good	Fair
Calco	9	1	2	F+	Fair	Good
Harmer	7	1	2	F+	Fair	Unsatisfactory
H. S. Laboratories	6	0	2	F+	Fair	Unsatisfactory
Geigy	7	0	3	F	Fair to poor	Unsatisfactory
Holland Anilin	6	0	2	F	Fair to poor	Good
Kohnstam	8	1	4	F	Fair to poor	Excellent
Providence	6	0	2	F	Fair to poor	Good
Dealer A	7	0	3	F−	Unsatisfactory	Excellent
* Dicks, David	8	0	6	U	Unsatisfactory	Unsatisfactory
Medicinal:						
Calco	7	5	0	E−	Very good	Good
Dealer C	7	5	0	E−	Very good	Good
Williamsburg	6	3	0	E−	Very good	Excellent
Coleman and Bell	7	4	0	G+	Very good	Good
Heyl	2	0	0		Very good	Not tested
Providence	9	2	0	G+	Very good	Unsatisfactory
Goldin	10	2	0	G	Good	Fair
H. S. Laboratories	9	4	0	G	Good	Fair
Merck	8	0	0	G−	Fair to good	Unsatisfactory
* Dealer A	7	0	2	F	Fair to poor	Excellent
* Harmer	8	1	5	F−	Unsatisfactory	Fair
* Mallinkrodt	9	0	5	F−	Unsatisfactory	Unsatisfactory
* Dealer B	5	0	3	F−	Unsatisfactory	Good

* These samples dissolve milk smears when used by the Breed method.

The results are listed in table 3, in which the same set of symbols is used as in table 1. A summary of the results is given in table 4. The most apparent fact is that the best results in staining have been obtained with the zinc-free dye. This is presumably because of the insolubility of the zinc salt in alcohol,

as Macy and Robertson, who used aqueous solutions, obtained essentially as good results with the zinc salt. The striking exceptions to the inferiority of "methylen blue for bacilli" are the samples from Coleman and Bell, and from the Heyl Chemical Company. After completing the test, it was learned that these two samples, although not labelled medicinal, and not claiming to meet the U. S. P. requirements, are fairly free from zinc, having been especially purified and corrected for bacterial staining.

Of the samples of medicinal methylen blue, six stand at the top, as a stain, with the honors quite evenly divided between them: namely, Calco, dealer C, Williamsburg, Coleman and Bell, Heyl, and Providence. Almost as good are the Goldin and H. S. samples. Of these eight companies, we find that the Calco Chemical Company, the Coleman and Bell Company, the Heyl Laboratories, the Providence Chemical Laboratories, and the H. S. Laboratories are making special efforts to put out a pure product well adapted to both staining and therapeutic uses. It is plain that they are succeeding in this, so far as staining properties are concerned, and provided the quality of their products is maintained any of them may be used as a stain without question.

One fault found with certain samples of methylen blue is that they have a tendency to dissolve milk smears off the slide when used for the Breed method. The chemical explanation of this phenomenon has not been obtained. The samples giving this trouble were the zinc salt sample from Dicks, David and Company, and the medicinal samples from Harmer Laboratories, Mallinkrodt Chemical Company, and from dealers A and B. It is interesting to notice that these same samples have been graded lowest according to their staining properties. They are plainly not to be recommended.

There is little correlation between the results of the staining tests and those obtained by Levine with the eosine-methylen-blue medium. The samples from dealer A, for instance, which stood low as regards staining properties were among the four

best samples in this medium. The medicinal sample from the Providence Laboratories, on the other hand, proved good for staining but unsatisfactory in this medium. The five samples grading good or very good as stains and also proving good or very good as an indicator were the two Coleman and Bell samples and the medicinal samples from the Calco Chemical Company, Williamsburg Chemical Company, and from dealer C. Those samples proving unsatisfactory were condemned because of toxicity. Strange to say, as many medicinal samples as zinc salt samples proved toxic, although for medicinal purposes the zinc salt alone is condemned on account of toxicity. In fact, for this medium, the zinc salt seems to prove almost if not quite as good as the free chloride.

There can be no question that, so far as our data go, one of the five samples just mentioned as satisfactory for both purposes should be recommended. The Heyl samples seem to rank with these as to staining properties, but for the reasons mentioned in the introduction, could not be included in the early tests, so we do not know how satisfactory they would prove in the Levine medium. We have not learned the original source of the sample from dealer C. The Williamsburg sample was labelled medicinal, but proves not to be entirely zinc-free. As a result, the composition of this product may well have been changed by the present time to meet U. S. P. requirements, and one could not count on getting another sample like the one tested. The two Coleman and Bell samples and the Calco medicinal sample all give very good results, and are produced by companies we believe to be reliable and to supply nothing but distinctly American products. Both companies we understand to manufacture their own methylen blue from American-made crudes or intermediates. Any of these three samples can be used with entire confidence of obtaining good results. Of these three, the Coleman and Bell "methylen blue for bacilli" has two points especially in its favor: it is not quite so expensive as the U. S. P. preparations, and it is the best of these three samples in the Levine medium.

GENTIAN VIOLET

The term gentian violet is at present very indefinitely used. Grübler apparently originated it, applying it to a certain mixture of dyes all closely related chemically and having similar staining properties. These dyes belong to the pararosanilin series. The pararosanilin base is considered to have the formula:

$$H_2N{\cdot}C_6H_4{\cdot}C(OH)\begin{cases} C_6H_4{\cdot}NH_2 \\ C_6H_4{\cdot}NH_2 \end{cases}$$

In this formula it will be noticed that there are six hydrogen atoms attached to the three amino-nitrogen atoms. These six hydrogen atoms may each be replaced by a methyl group (CH_3), and ethyl group (C_2H_5) or even by a benzyl group (C_6H_5). The compounds of most importance in gentian violet are those with four, five and six methyl groups respectively, known as:

tetramethyl-pararosanilin
pentamethyl-pararosanilin
hexamethyl-pararosanilin

In these three compounds, the greater number of methyl groups present the deeper is the shade of violet. Thus the tetramethyl compound is a reddish violet while the hexamethyl compound is a bluish violet. The introduction of a benzyl group tends to darken the shade still further. The tetra- and pentamethyl compounds are seldom prepared in a pure state, but the hexamethyl compound, under the name of crystal violet, is readily obtainable in fairly pure form. Grübler's gentian violet, besides these compounds, is supposed to have contained other rosanilins, but these three methylated pararosanilins were the most important constituents. American gentian violets are sometimes pure crystal violets, sometimes some other dye of this series, and sometimes various mixtures of the above mentioned dyes. The term gentian violet is not used by the dealers in textile dyes nor by their dye chemists. This stain is ordered only by the biologist, and hence it is furnished only by biological supply houses and some dye manufacturers that specially cater to the biologist.

These stains may be grouped in the following three categories:

I

Methyl violet
Methyl violet B
Methyl violet 2B
Methyl violet BS
Methyl violet BN
Methyl violet BO
Methyl violet BBN
Dahlia B

Under these designations are sold certain mixtures of the tetramethyl and pentamethyl compounds, in varying proportions, sometimes also containing the hexamethyl compound as well. The letters following the name are used in the trade to indicate the shade, methyl violet B and methyl violet 2B, for example, each being progressively deeper in shade than plain methyl violet. The trade designations do not refer to chemical composition except in so far as the deeper shade indicates a more highly methylated compound.

II

Benzyl violet
Methyl violet 5B
Methyl violet 6B
Methyl violet 7B

These dyes are benzylated compounds, generally mixed with some of the purely methyl compounds above mentioned. The number of B's in the trade designation indicates the depth of violet produced in dying.

III

Crystal violet

This dye is the pure hexamethyl compound. It is the most readily obtained in pure state of the whole group, and there is less confusion in regard to its composition than in regard to any of the others.

For the present series of tests, samples of the following were collected: Methyl violet, methyl violet B, methyl violet 2B, methyl violet BS, methyl violet 6B, crystal violet, and gentian violet. There were over thirty samples in the entire lot, too many, it was felt, to send to any one investigator for the first test. Accordingly a more or less indiscriminate selection was made, so that each investigator had about twelve samples to test, generally one or two of each type of dye. They were asked to use the samples for the Gram stain, each man to employ his own technic, but to give the exact procedure used. As it turned out, scarcely any two men used the same technic, so the results varied greatly. The plan is to run another test with a standard technic. At present so few men have reported on any one sample of dye and such various technics were used that the results are not regarded as having more than a general significance. It would not be fair to judge the individual samples on the basis of this preliminary work; so in this report the names of the manufacturers and distributors are not given. Each sample is denoted by number alone, each number indicating a certain business house. Results are given in table 5.

It was not expected when the work was started that the compounds of lower methylation would prove satisfactory for the Gram stain. It was somewhat of a surprise, therefore, to find that out of 84 tests of individual samples of methyl violet. methyl violet B and methyl violet 2B, there were 28 reports of good results, and one report of excellent results. Nevertheless, out of these same 84 tests, there were 36 reports of unsatisfactory findings, such a high percentage in comparison with the results obtained with the higher methylated compounds that no further work is to be done with these dyes.

The dyes called anilin violet, methyl violet BS, methyl violet 6B, and crystal violet, give in general as good results as "gentian violet" and sometimes better. There is some variation, however, in the products of different manufacturers, but on the basis of these few tests, none can be condemned. According to present indications, crystal violet can be substituted very successfully for gentian violet in the Gram stain. Some manufacturers at

TABLE 5

Report of individual investigators on the samples submitted for use in the Gram stain

DYE	MANUFACTURER'S NUMBER	HOCHTEL	HARRISON	STOVALL	GRADWOHL		FLEISHER		MURRAY	EISENBERG	HUCKER		ROOS	HANNUM	HARKINS	GORHAM			HOFFMAN	AVERAGE GRADE
					Stirling solution	Carbolic solution	Stirling solution	Carbolic solution			Stirling solution	Carbolic solution				Stirling solution	Carbolic solution	Atkins method		
Anilin violet	62						U	U	G	U	E	G				G	E	E		G−
Methyl violet	27		F	G	F	U	F	G	G		F	F								F+
	62	U									F	F							G	F
	85	G	U		F	G	F	F		·	U	U								F
	123										U	F				U	G	G		F
	127		G	G	U	U				U	U	F								F−
	223			U							F	G							G	F+
	229									U	U	G							G	F−
Methyl violet B	77			U					F	U	F	F							G	F−
	228									U	U	U							G	U
Methyl violet 2B	77	G			U	U					G	G								F+
	123	G	F	U			G	U			F	F							G	F
	192						F	U	G		E	G							F	G−
	196		U				U	U			F	G							F	F
	223									U	U	G								F−
	228			G					G		U	U							U	F−
	230		G	U						U	U	U				U	G	G		F−

| | | |

present make this substitution for us, sending crystal violet when gentian violet is ordered.

The chief cause of variation in results seems to be due to the differences in technic. This shows plainly the need of standardizing the Gram stain, a task proposed to this committee some time ago. It is hoped as the result of the present work to accomplish this. Apparently the Stirling technic does not always work with these American products, as they are generally more concentrated than Grübler's gentian violet. Grübler's stain is well known to have contained a large amount of dextrin. The investigators who have used one of the methods calling for a carbolic solution seem to have obtained the best results; although one man who has compared a carbolic solution with the anilin-sulphate solution of Atkins, reports even better results with the latter.

In conclusion it can be said that several of the American gentian and crystal violets are as good, and some of them better than the Grübler product, when the Gram stain is used as a criterion. It is still too soon to endorse the product of any one manufacturer or distributor, but good results can apparently be counted on with any of the following:

Methyl violet 6B	Coleman and Bell, Heyl, or H. S.
Crystal violet	Coleman and Bell, Harmer, or Providence
Gentian violet	Coleman and Bell

It is suggested that anyone wishing to order before definite recommendations are made by the committee specify one of these seven products.

GENERAL RECOMMENDATIONS

There is at present so much confusion in this field that the biologist generally has little idea what actual dye he is buying under any given name. One practice that has contributed to this is that distributors buy the stains in bulk and then repack in small containers bearing their own label without mention of the name of the manufacturer or standardizer. Sometimes the distributor renames the stain on his own responsibility. There

is no objection to the distributor's name occurring on the label, provided the stain is designated by the original name, followed by the name of the manufacturer or standardizer from whom it was obtained. Our chief recommendation at present is therefore that everyone buying stains from dealers in general laboratory supplies insist that the source of the stain as well as the distributor's name be printed on the container. It is our hope that the distributors may coöperate with us in doing this.

When it comes to selecting between the different brands of stains on the market, it must be recognized that the number of dyes so far tested are so few that no general recommendations can be made as yet to apply to all bacteriological dyes. The present work, however, indicates that the products of Coleman and Bell, the Calco Chemical Company, the Providence Chemical Laboratories, and the H. S. Laboratories, all rank well. The managers of the companies are willing to coöperate, and they are all putting on the market stains that there is every reason to believe are of strictly American make. In particular, we would call attention to the extremely good showing of the Coleman and Bell (formerly National Stain and Reagent Company) products. This company, moreover, has been good enough to furnish the committee with fairly definite specifications for those dyes so far tested, an important point because one of the chief matters to be kept in view is the permanence of the supply of stains. There is every reason to expect that the Coleman and Bell Company, given adequate support by the users of stains and government protection against foreign products, will be able to continue in the business; but if it should not, much of the information obtained by this company would be at the disposal of our committee to use in duplicating their products. On account of their willingness to coöperate and their eagerness to be of service to bacteriologists, all of the Coleman and Bell products—even those not yet tested—deserve a thorough trial.

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